

THE REACTION OF HYDRA TO INANITION.

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During the past fifteen years the senior author has observed that Hydra, kept in laboratory aquaria to which food has not been added, decreased greatly in size. In this general or casual observation, it was further noticed that green Hydras survived under these adverse conditions for much longer periods than did Hydras of other species. This has been a general experience in this laboratory, though it stands in sharp contrast with Wagner's ('04) statement that "Green Hydras stand starving very poorly, usually perishing in two or three days" (p. 612). In later years it was observed further that the poorly fed Hydras would lose parts of their tentacles. Sometimes the full complement of tentacles has disappeared. During the last three years, we have been seeking to learn the conditions under which Hydras lose their tentacles.

The literature presents the following causes by which Hydras lose their tentacles. N. Annondale ('07), in studying *Hydra orientalis*, found a seasonal variation from four to six tentacles. During the hot season this Hydra has but four tentacles, while during the cold part of the year it has six tentacles. G. Entz ('12) observed that an infection with *Amæba hydroxena* may lead to a complete loss of tentacles. E. Reukauf ('12) observed that the ciliate *Prodon teres* caused Hydra to lose its tentacles. Again, the presence of another ciliate, *Kerona pediculus*, according to P. Schultze ('13), leads to hypertrophy of the tentacles. E. Schultz ('06) observed that hunger set up a process of dedifferentiation within the tentacles of Hydra; just as Huxley and De Beer ('23) found that hunger and toxins caused dedifferentiation and resorption of the tentacles of *Obelia* and *Campanulana*. Huxley and De Beer observed that this process of dedifferentiation and resorption might involve, not only the tentacles, but also part of the zoöid. Finally Berninger ('10) observed that, in response to inanition, Hydra lost its tentacles; but he did not see the manner in which they were lost.

We have found that *Hydra* is very susceptible to the impulses arising from scarcity of food. *Hydras*, at the time they are taken from the pond, present sometimes one or more fragmentary tentacles. That these do not represent mere accidental losses is suggested by the fact that in a flourishing population of *Hydras* one does not readily meet with such imperfect specimens, whereas when the population of a pond is on the decline specimens with incomplete tentacles are quite frequently encountered.

That *Hydras* react rather readily to hunger is further indicated by the observations of Wilson ('91). He records that "After the exhaustion of the food-animals—which takes place rapidly when the *Hydras* become very numerous—the *Hydras* are compelled to live wholly upon mud" or sediment at the bottom. In this sediment they find fragments of plant tissue and numerous minute Infusoria. The polyps, "after descending, usually gorge themselves with the sediment at the bottom." Welch and Loomis ('24) verified this observation. They found, however, "that the *Hydras* did not always thrive indefinitely on the sediment material dredged from the deepest parts of the lake," p. 227.

Realizing, then, the susceptibility of *Hydra* to hunger, we set to work to see if inanition was a factor leading to the loss of tentacles. This effort was made through the observation of living isolated individuals and the histological study of isolated individuals and controls.

Hydra has been observed taking into its body a tentacle's end as it clung to the food. This has been seen frequently. For example Mr. J. W. Nuttycombe saw a *Hydra* swallow the ends of two tentacles that had been involved in the capture of a Cyclops. After considerable struggle on the part of both the tentacles and the body-proper, these tentacles were thrown from the coelenteron. So far as could be determined these tentacles had not been injured. In such examples, an effort to free the tentacle from the coelenteric cavity is always apparent.

In contrast to this, Mr. M. C. Yoder and the senior author observed an isolated *Hydra*, that had been deprived of food, hold a tentacle within the coelenteric cavity for twenty minutes. Throughout this period the tentacle remained quiet. There was

in this case no effort to free the ingested tentacle. After twenty minutes, however, the tentacle was seen to be slipping out of the mouth. When freed the tentacle presented a constricted zone about which the peristome had held it during the period of ingestion.

The history of this tentacle has an interesting bearing upon our observations; for it shows that the tentacle was not one that had undergone necrosis prior to its having been ingested, since it showed no eroded tissue and eventually fully recovered, leaving no trace of the constricted region. This tentacle, therefore, had not suffered necrosis before being ingested. Marshall ('23) makes this observation: "Some brown Hydras were induced to swallow pieces of *Hydra viridis* by slipping the latter inside the carapace of *Daphnia*, but they were ejected along with the remains of food," p. 614. The suggestion may be taken that only dead Hydra-tissue might be freely ingested since Marshall, in this case, saw fresh Hydra tissue "ejected along with the remains of food."

But our observations do not lend support to this negative inference. Miss Edna McNally in 1923 observed an *Hydra viridis* "put the end of one of its tentacles into its mouth." The end of the tentacle could be clearly seen inside of the cœlenteron. After the tentacle had been kept in the cœlenteron about fifteen or twenty minutes, the Hydra dragged the shortened tentacle with a ragged end from the mouth, leaving part of it within the cœlenteron.

In addition to having actually seen an Hydra ingest a piece of its tentacle, we have observed a green specimen, which during sustained inanition had but recently lost parts of some of its tentacles, throw out from its mouth defragmented tissue of green Hydra. On one occasion, after such cellular material had been regurgitated, it was again ingested.

Thus, actual observation of living individuals has yielded evidence that Hydras, that have been deprived of food, feed upon their tentacles.

Further evidence was secured through isolating two series of individuals. By depriving one of these series of food and feeding the other series well, we have secured some interesting results.

On March 14, 1925, six individuals were isolated in six watch

glasses and given no food. Another lot of six were isolated. These were used as controls and were kept supplied with food in the form of *Cyclops* and daphnids. The observations were kept up until April 13, during which time, one of the controls had died (March 22) but none of these controls had lost part of their tentacles. Except for the one specimen dying, the controls at the end of the period of observation were in excellent condition and showed no negative features. The starving specimens presented a marked contrast to the control series for by March 22 two specimens had died. Of these two that had died, one had lost more than two thirds the length of each of its tentacles the day before it had died. The four other specimens, by April 14, had lost parts of their tentacles. The most conspicuous individual in the starved series was designated specimen six in our records. Figure 5, *A*, represents the contour of this animal when expanded at the time of isolation. On March 24 it showed, in the expanded condition, that its tentacles were shorter and knobbed on the end (Fig. 5, *B*). After that not much change could be noted, except that the knobbed condition of the tentacles had disappeared, until March 30. Then it was observed that a great amount of the tentacles had been lost (Fig. 5, *C*). On March 31, but mere stumps of the tentacles remained (Fig. 5, *D*). The specimen on April 1, showed the bud of one tentacle making its appearance. By April 8, tentacles relatively as long as those shown at *A* in Fig. 5 were present. During the period of inanition the body, as a whole, had been reduced in size so that by April 10 the regenerated tentacles were relatively longer than those which were involved at the beginning of the observation (Fig. 5, *E*). On April 9, some parts of this second crop of tentacles had been eaten. On April 14, the specimen died.

In the above observations, no effort to control the factor, presented by the accumulation of toxic metabolic products, was made. It is quite possible that such effort need not have been made, for the fed Hydras had grown in size and, therefore, must have thrown a greater amount of toxic material into the water than did the unfed specimens. In addition to this, the presence of the *Cyclops* and daphnids added much to the concentration of metabolic toxins in the water of the fed specimens as compared

to the water in which the unfed specimens were kept. However, a second series of observations was made. In this series, five specimens were isolated and carried through unfed while five isolated specimens were fed to serve as controls. Each day the water in all watch-glasses containing Hydras was aerated by blowing air through it and every twenty-four hours or every forty-eight hours the old water was drawn off and fresh spring water added. In these instances, we got just as conspicuous contrast between the unfed series and the fed series as has been described for the series running from March 14 to April 13. The most pronounced example, obtained in this manner of Hydra's reaction to inanition, was obtained from specimen 4 of a series begun October 6, 1925, and ending October 30. A camera lucida drawing was made of this specimen as it hung from the surface of the water in the watch-glass, October 6 (Fig. 6). Three days later the camera lucida drawing shows that there had been a marked reduction in the size of the specimen (Fig. 6). On October 11, the specimen presented only mere stumps of tentacles (Fig. 6). Camera lucida outlines made daily showed that those tentacle stumps gradually grew until by October 22 the camera lucida outline reveals that the now smaller Hydra had regenerated seven tapering tentacles (Fig. 6). The next day, however, one of these tentacles had disappeared (Fig. 6). A second tentacle was missing October 24 (Fig. 6). Two days later the camera lucida drawing indicates that the specimen had lost the greater part of each of its tentacles (Fig. 6). In this condition the specimen remained as a listless individual for three days and then died.

The above specimens were examined daily for the presence of parasites such as *Amoeba* and other protozoa and were thus determined to have been free of such infection. The abruptness, however, with which a part of a tentacle or a whole complement of tentacles would be lost did not suggest that these tentacles disappeared through attack by parasites or by degeneration and resorption. It suggests rather that the specimens had been feeding upon their tentacles.

The histological evidence supports the idea that dedifferentiation, degeneration and parasites were not involved.

Specimen "369-2-a" had been isolated in a hanging drop until it had lost its tentacles. The slide made from this specimen bears a complete series of sections in which the shortened tentacles show no erosion such as *Amœba* and other parasites cause. Moreover, all of our slides show no dedifferentiation processes. We have slides that show wound scars at the end of tentacular stumps but in which the ectoderm is laid down as a continuous sheet about the tip (Example "370-2").

But our histological evidence leads further to the conviction that tentacle-material is ingested and digested. Mr. Looper, of this laboratory, has two individuals sectioned, in one of which the sections show a series of sections of three fragments of tentacles lying within the coelenteron, while the other shows but one fragment of tentacle lying within the coelenteron (Looper's slides "2 ovum la," and "Hydra Ovum 11").

The transverse sections of the tentacular fragments, that are to be found in sections of polyps that have suffered inanition, show a diploblastic pattern but present very poor detail. In Fig. 4 we have a transverse section of an ingested tentacular fragment. The region of the endodermal epithelio-muscular cells is most greatly broken down (Fig. 4, b). What we take to be residia of secreting endodermal cells are indicated at a. The ectoderm seems to be least attacked by the digestive fluids of the coelenteron. All elements here, too, have been greatly shrunken.

Not only do the histological specimens show the tentacular fragments lying within the coelenteron but they indicate that the material, thus taken in, is digested and absorbed.

This was further checked by fixing specimens by the pond in which a vigorous population of Hydras was growing and contrasting the histology of these with that of the isolated specimens that had been kept under daily observation in the laboratory. Slides, bearing material taken from the vigorous population of a pond, show no Hydra-tissue within the epithelio-muscular cells of the endoderm. In contrast to this, the histology of specimen "375" is significant. This specimen was isolated in a Petri dish, containing spring water, at 3:30 P.M., October 28, 1921. At 9:25 A.M. the next day it was found to have had but the merest

stumps of tentacles. Our slide shows the tentacular stumps involving but a few sections of the series, thus recording their small size. Hydra-tissue, being digested within the coelenteron, is found in these sections. While within the endoderm's epithelio-muscular cells are *many* nematocysts.

Another specimen, "369-1," was kept isolated within a hanging drop for three days and examined daily. At the end of this period, it was found to be in a listless condition and to have very short tentacles. This Hydra, in the fixed and sectioned condition, revealed many nematocysts within the coelenteron and food-vacuoles of the epithelio-muscular cells of the endoderm.

Specimen "380" was isolated in a test tube. This tube was kept partly submerged in the pool from which the Hydra had been taken. Daily inspection revealed that on the seventh day the tentacles were blunt, their ends ragged, no amoebas or other parasites present. The series of sections on our slide 380 shows almost wholly digested hydra-tissue within the coelenteron, there being left, as yet, naked nuclei, at one side of the dissolving mass, and a cnidoblast, in bad condition, surrounding a "stinging" nematocyst. In addition to this there are many nematocysts in various phases of digestion within endodermal cells.

These observations indicate that, not only are the protoplasmic constituents of the ingested fragments of tentacles digested and absorbed, but that even the nematocysts are likewise appropriated as food.

The fact that these structures can be digested by Hydra is interesting. Glaser and Sparrow ('09) found that "in the case of peptic digestions, all the tissues except the nematocysts, were dissolved," p. 362. But it has since been observed that parasitic ciliates and amoeba can digest the nematocysts of Hydra. The histology of Microstoma's enteron or "intestine" greatly resembles that of Hydra and it does not digest the nematocysts but hands them over for offensive or defensive purposes to the mesenchyme. Hydra's immediate demand, during inanition, is neither defensive nor offensive but nutritional, so it digests the nematocysts. Fig. 1 shows a nematocyst from which the cnidoblast has already disappeared. Except for the lacking cnidoblast the nematocyst, as it lies within the food vacuole of

an endodermal epithelio-muscular cell, does not present a markedly unusual appearance. In Fig. 2, however, the enclosed thread of the nematocyst displays marked shortening and the nematocyst, as a whole, takes the stain less readily. In Fig. 3, we have a contrast presented between a nematocyst that has been almost digested and one lying in the usual position in the ectoderm. It is seen here that the nematocyst within the endodermal cell has been almost wholly dissolved. Eventually no trace of the ingested nematocyst is to be found. The hungry *Hydra*, therefore, consumes not only the readily digestible tissues of its tentacles but also the nematocysts. From the material, thus obtained, energy is derived upon which the life of the polyp is tided through a period of inanition and by which the polyp is enabled to develop a new complement of tentacles.

An interesting feature of this process is presented in the fact that the stumps of tentacles receive relatively much more of the ingested tentacular material than do other parts of the body. Our slide "375" shows this clearly. This slide bears in series the sections of an *Hydra* that had been isolated in a Petri dish in the laboratory for eighteen hours. At the end of this period but small stumps of its tentacles had remained. In this series of transverse sections there are ninety-five sections.

The tentacles involve about twenty of these (10 microns) transverse sections. The endodermal cells in the sections that show the bases of the tentacle-stumps are heavily charged with food-vacuoles that contain nematocysts and *hydra*-cells. The endodermal cells of the tentacle-stumps have in this instance much more food supplied them than do the endodermal cells of the body proper. This is in contrast to what ordinarily takes place. If a complete *Hydra* be fed and later sectioned, the endodermal cells of the tentacles will be found to have relatively fewer food-vacuoles than the endodermal cells of the body-proper. So here again we have it suggested that in a diploblastic animal local needs must be locally met. In *Hydra*, having but two tissues there can be no circulatory medium. Therefore, when material is needed for the regeneration of tentacles it cannot be taken up by the general endoderm of the body-proper and then as lymph or plasma sent to the tentacular bases, but the

material must be handed directly, in the form of food, to the endoderm of the tentacle-bases.

SUMMARY.

Hydra, as a diploblastic animal, can have no circulatory medium. Perhaps for a similar reason it can have no storage tissue such as fat. Green Hydras seem able to fall back upon the surplus foods of its zoöchlorellæ, for, contrary to some observers, it has been our experience that *Hydra viridis* is much less influenced by inanition than other species.

In all species, if the inanition be prolonged and the individuals be kept free from parasites and concentrated toxins, each specimen not getting food, will begin to feed upon its tentacles. First the ends of the tentacles are bitten off. If now food is yet withheld from the Hydra, it will feed upon its tentacles until but the merest stumps are left.

The ingested tentacles will be digested. Even the nematocysts (both types) will be taken into food-vacuoles within the epitheliomuscular cells and be completely digested and absorbed.

After the Hydra has thus fed upon and appropriated its tentacles—except for mere stumps that stand about the peristome—the bases of the amputated tentacles will regenerate and the Hydra, now reduced in size, will possess a new group of tentacles.

In feeding upon the tentacles that have been ingested, the endoderm of the stumps of tentacles appropriate relatively more of the ingested tentacle-material than is appropriated by the endoderm of the body proper. This appears to be correlated with the fact that regeneration is to take place in the tentacular stumps.

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EXPLANATION OF PLATE.

FIG. 1. Interlocking secreting (a) and epithelio-muscular (b) cells of the endoderm. b bears a food vacuole (c) within which is a nematocyst that shows little distortion. $\times 1500$.

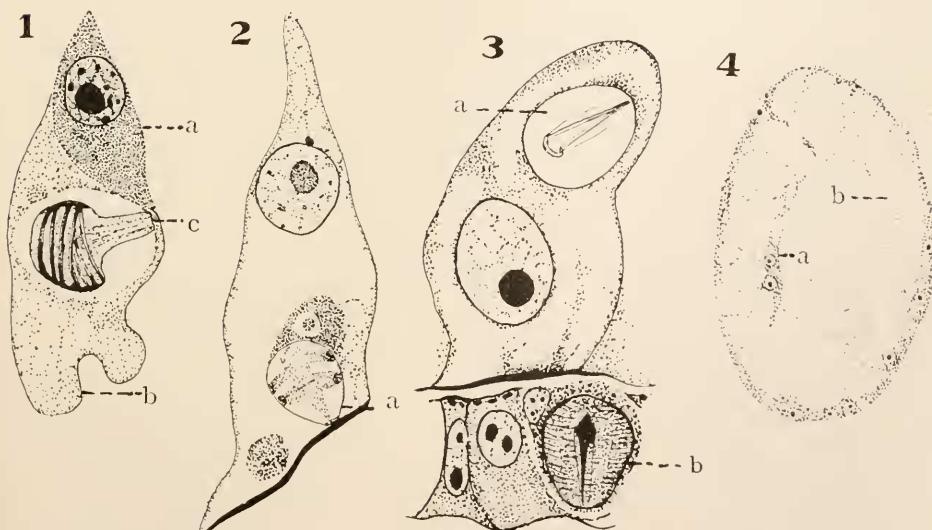
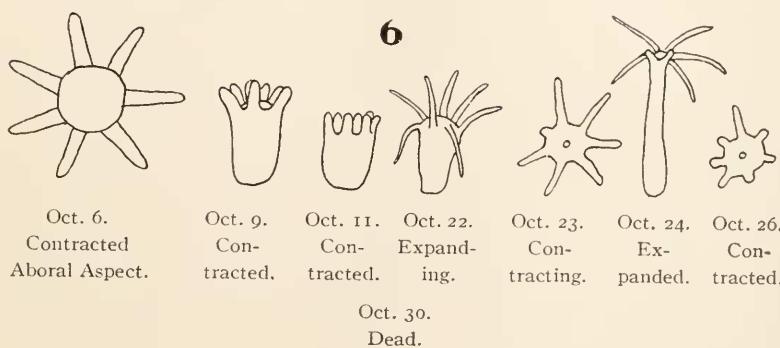
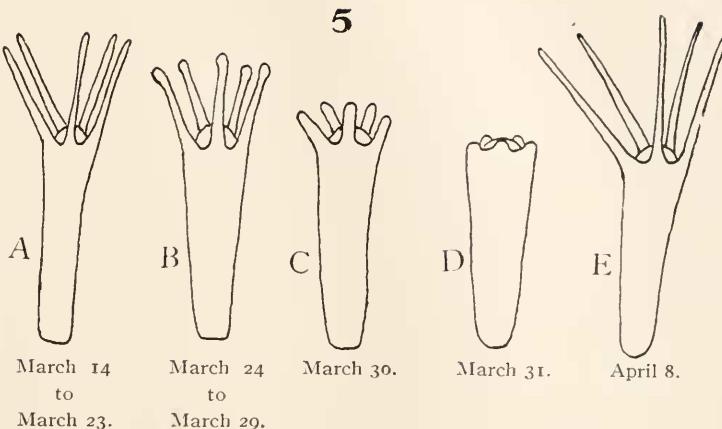
FIG. 2. An epithelio-muscular cell of endoderm bearing a food vacuole (a) within which is a nematocyst that shows advanced internal disintegration. $\times 1500$.

FIG. 3. An epithelio-muscular cell of endoderm with adjacent ectodermal cells. A greatly broken down nematocyst within a food vacuole (a) is contrasted with a nematocyst in the usual position within the ectoderm (b). $\times 1500$.

FIG. 4. One of the transverse sections of a tentacular fragment that lay in the coelenteron of a sectioned *Hydra* from which food had been withheld. (a) residia of secreting endodermal cells; (b) residia of epithelio-muscular endodermal cells. $\times 750$. From H. L. Firebaugh's slide "No. 15. 34.7-85.8."

FIG. 5. Series of free-hand outlines to indicate changes that had taken place in an individual *Hydra* from which food had been withheld.

FIG. 6. Series of camera-lucida outlines to indicate changes that had taken place in an individual *Hydra* from which food had been withheld.



CILIATED PITS OF *PRORHYNCHUS STAGNALIS*.

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The specimens used in this study were collected in the early months of the year in a very small, slowly running stream, the outlet of a spring, which is located about a mile west of the University of Virginia. They occurred in greatest abundance from twenty-five to forty feet below the spring, in the soft, oozy, decaying organic matter at the bottom of the stream. *Prorhynchus appplanatus* was always found in equal, if not greater numbers, along with *Prorhynchus stagnalis*.

Some of the grass, weeds, and algae growing in the stream were also taken along with the sediment, and the whole collection placed in a large aquarium dish and left to stand for from twelve to twenty-four hours. The rhabdocoëles appeared at the edge of the aquarium, near the water's surface, sometimes just beneath the surface film. When a film of bacteria developed at the surface of the water, the animals soon died, seldom being obtained five days after the collection had been in the laboratory. By keeping some of them in small dishes in spring water with some small amount of the decaying matter collected in the stream, and by adding fresh water every three or four days, specimens were kept alive for a maximum period of two weeks.

The body of *Prorhynchus stagnalis*, when moving through the water, is very slender or thread-like, being about the same width all along the body-length. When at rest, the posterior half is slightly broader, the average length of the body being about 6 mm.

The anterior region of *Prorhynchus stagnalis* is the exploratory region of the body. For, in response to a disturbance, the region of the body, anterior to the ciliated pits, of an animal that is quiet or of one that is creeping, is laterally expanded (Fig. I, *l.e.*) and swayed to and fro; while in addition to this the cilia of the pits, during disturbance, are set into active motion. The cilia of the ciliated pits are quiet when the animal is not excited or disturbed.

For histological study, specimens were fixed with greatest success in Bouin's fluid, cut from five microns to seven microns thick, and stained in iron haematoxylin, with Bordeau red or eosin as a counter stain. It was found best to handle the specimens in very small numbers at a time. While fixing, it was also found advantageous to keep the animal under the slight pressure of a cover glass to prevent the curling of the body. The best stain for differentiating nervous tissues was Mallory's connective-tissue stain, fixation being in Zenker's fluid. The nervous tissue is stained blue in this case.

Acknowledgment must be made to Dr. W. H. Taliaferro for a box of slides of *Prorhynchus stagnalis* which he left with Dr. Kepner, and which were of great aid in this work.

It is necessary to consider the central nervous system with which the two ciliated pits are associated before discussing the latter. There are two dorsal ganglia just anterior to the opening of the muscular pharynx as it lies at rest within the pharyngeal sheath (Figs. II and III, *d.g.*). These ganglia are connected across the pharyngeal sac by a relatively broad commissure (Figs. II and III, *d.c.*). Posteriorly from the dorsal ganglia extend latero-ventrally two nerves, which cannot be traced beyond the beginning of the enteron (Figs. II and III, *p.n.*). Anteriorly from the dorsal ganglia extend latero-ventrally two other nerves, which lie in contact with the mesial surfaces of the ciliated pits, and passing beyond the ciliated pits branch in the extreme anterior portion of the body (Figs. II and III, *a.n.*). There is another set of nerves connected with the pharynx, the exact path and relation of these nerves to the ganglia and pharynx having not been definitely determined.

From Kepner and Taliaferro's ('16) paper on "Organs of Special Sense of *Prorhynchus applanatus* Kennel," we find this comparison of the ciliated pits in *Microstoma caudatum* and *Prorhynchus applanatus*:

"As stated before, we consider the ciliated pits the chief organs of special sense. These organs open on the ventral side and are disposed laterally and ventrally to the thick nerve commissure which joins the two dorsal ganglia. . . . When the animal is crawling upon a surface, it seems to make numerous exploratory

movements by raising and lowering the anterior sixth of its body. Thus we have a crawling animal with ventrally disposed ciliated pits which makes its exploratory movements by raising and lowering the anterior end of its body. It is well to compare the conduct of this animal with that of *Microstoma caudatum* Leidy with reference to the position of their respective ciliated pits. *Microstoma caudatum* is a free-swimming animal and has laterally disposed pits. As we have shown ('12) this animal makes exploratory movements by moving its anterior end from side to side. Likewise, we gave experimental evidence to show that these exploratory movements were made in order to test the surrounding medium. Thus we see by the comparison of the two rhabdocœles that the method in which they test the surrounding water conforms to the position of their ciliated pits."

So much for *Microstoma caudatum* and *Prorhynchus appplanatus*. Kepner and Taliaferro in *Microstoma caudatum* dealt with a form that was primarily a free swimming one; in *Prorhynchus appplanatus* with a form that was primarily a creeping one. *Prorhynchus stagnalis* usually creeps. It is, however, provided with mucous cells at the posterior end of its body by means of which it can attach itself to the substratum. From this stationary point it can move its body about in an almost complete circle, sometimes adhering closely to the surface, but frequently lifting the anterior third of its body, moving it either from side to side or backwards and forwards. Therefore, it would be natural to expect its special sense organs to be ventro-laterally disposed—and such happens to be the case. In *Prorhynchus stagnalis* is, therefore, presented a form that sometimes creeps and sometimes swims freely. So it is of interest to observe that when it is creeping the anterior end, through flattening, applies the ciliated pits to the substratum much as *Prorhynchus appplanatus* does; whereas when the animal moves or sways through the water, its anterior end, through becoming rounded, places the ciliated pits more laterally as they are always placed in *Microstoma caudatum*. It has been thus observed that the position of the ciliated pits of *Prorhynchus stagnalis* also conforms with the diverse habits of *Prorhynchus stagnalis*.

The pits are directed obliquely posteriorly and mesially, there being a decided curvature in the "neck" of the pit.

In the histological structure of these pits we define three regions; the neck or transitory region (*t*), the sensory region (*s*), and the glandular region (*g*) (Figs. IV, V, and VII). All the components of the pits, with the single exception of the muscle fibers (Figs. IV, VI, and VII, *l.m.*, *m.m.*), which are attached to the pits, are considered to be of ectodermal origin. This assumption is based upon the fact that Kepner and Taliaferro ('12) in their study of the ciliated pits of *Microstoma caudatum*, clearly showed that in a newly-forming individual, both the glandular and sensory cells developed as modifications of an invagination of the general body epithelium or ectoderm (Figs. IV, V, and VII, *g.b.e.*). The pits of *Prorhynchus stagnalis* measure from twenty-five to thirty-five microns in length and from twelve to fifteen microns in width, (excluding the glandular cell, which is about two thirds of the length of the pit proper), being narrower near the mouth in the fixed condition on a slide than at the fundus of the pit. This latter condition, that of the narrowness of the mouth of the pit, is in sharp contrast to the contour of the functioning pit.

With the exception of the glandular region, the ciliated pit is, like the rest of the body epithelium, a syncytium, with its nuclei specialized to conform to their respective functions. The presence of a glandular cell makes even stronger the belief that the pit is a gustatory organ.

The nuclei of the ciliated, transitory epithelium are eight in number, a double row of four nuclei, one row being disposed obliquely dorso-ventrally, forming a part of the lateral wall of the pit, while the other row forms a similar mesial wall. These nuclei have an elongated, ovate contour with the axis of each nucleus in a plane that is at right angles to the axis of the pit, and bent to conform to the curved wall of the pit (Figs. IV, V, VI, and VII, *t*). They are densely granular, and the aspect of the possible fifth pair of nuclei nearest the mouth of the pit gradually fade into the appearance of nuclei of the general body epithelium.

There are also two rows of sensory nuclei, four in each row, and placed in the same plane as the nuclei of the transitory cells (Figs. IV, V, VI, and VII, *s*). On the inner walls of the pit next to the sensory nuclei are two rather densely granular ridges,

(Figs. IV, and VI, *r.*), presumably for the receiving of stimuli. These ridges are devoid of cilia, though the remaining inner surface of the pit is highly ciliated. Due to the difficulty in differentiating nervous tissue very clearly through any of the simpler staining methods, it was impossible to distinguish any special nerve endings. The main anterior nerves from the dorsal ganglia were closely applied to the outer surface of the pit along its mesial side only (Figs. IV, V, VI, and VII, *a.n.*). Until better stained sections can be studied, the matter of nerve application to the pit will have to rest with this general statement.

At the base of the lumen of the pit, and connected with it only through small pores (*p.*) which pass through the syncytial wall of the pit on the anterior angle of the fundus lies* a large uni-cellular gland (Figs. IV, V, VI, and VII, *g.*). Its contour is very irregular, the cell giving off a number of processes. There are two interesting features to this glandular cell not noted in that of *Prorhynchus appplanatus*. One concerns the characteristic bent, ovate contour of the nucleus of this cell (Figs. IV, V, and VII, *g.n.*), with clearly defined chromatin granules. The other interesting fact concerns the presence of a simple drainage system in the form of intra-cytoplasmic canaliculi (Figs. IV, V, VI, and VII, *cn.*), which pour the mucus-like secretions of the cell through the pores in the syncytial wall into the pit (Figs. IV, VI, and VII, *p.*). These canaliculi have been observed both in an empty state and filled with materials of secretion.

Connected with the ciliated pits are several non-striated muscle fibers, arising both laterally and mesially, and passing closely along the surface of the glandular cell. There is only one fiber attached to the lateral wall (Figs. IV and VII, *l.m.*), but at least three fibers attached to the mesial wall of the pit (Figs. VI and VII, *m.m.*).

SUMMARY.

1. The correlation between the position of the ciliated pits in *Prorhynchus stagnalis* and the way in which the anterior portion of the body makes its exploratory movements is met in this rhabdocoel in the same manner as in *Microstoma caudatum* and *Prorhynchus appplanatus*.

2. The ciliated pits of *Prorhynchus stagnalis* are composed of a

definite number of cells, seventeen each. In spite of the fact that *Prorhynchus stagnalis* has no specialized sensory tissue in the form of a pair of simple eyes, *Prorhynchus stagnalis* is considered being more highly developed in that a much larger number of cells go into the formation of the pits than in both of the sensory organs of *Prorhynchus appplanatus*.

3. The ciliated pits of *Prorhynchus stagnalis* are sharply differentiated into three regions: transitory, sensory, and glandular.

4. There are two interesting features to the glandular cell not noted in *Prorhynchus appplanatus*: (1), the characteristic curvature of the nucleus; and (2), the intra-cytoplasmic canaliculi.

5. The ciliated pits of *Prorhynchus stagnalis* possess a definite musculature, attached to both their mesial and lateral walls.

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EXPLANATION OF FIGURES.

PLATE I.

FIG. 1. Ventral aspect of *Prorhynchus stagnalis* to show especially the lateral expansion (*l.e.*) of the exploratory region anterior to the ciliated pits (*c.p.*). Chitinous penis (*ch.p.*); pharyngeal sac (*ph.s.*); pharynx (*ph.*); penis bulb (*p.b.*); enteron (*en.*); seminal vesicle (*s.v.*); vas deferens (*v.d.*); egg about to be liberated (*e.*); ovary (*ov.*); testes (*t.*); opening of female reproductive organs (*♀*); (Modified from Von Graff).

FIG. 2. Dorsal aspect of anterior third of body to show relation of ciliated pits (*c.p.*) to central nervous system. Pharyngeal sac (*ph.s.*); pharynx (*ph.*); dorsal commissure (*d.c.*); right dorsal ganglion (*d.g.*); right anterior nerve (*a.n.*); right posterior nerve (*p.n.*).

FIG. 3. Right lateral aspect of anterior third of body to show relation of ciliated pit (*c.p.*) to central nervous system. Pharyngeal sac (*ph.s.*); pharynx (*ph.*); dorsal commissure (*d.c.*); dorsal ganglion (*d.g.*); anterior nerve (*a.n.*); posterior nerve (*p.n.*).

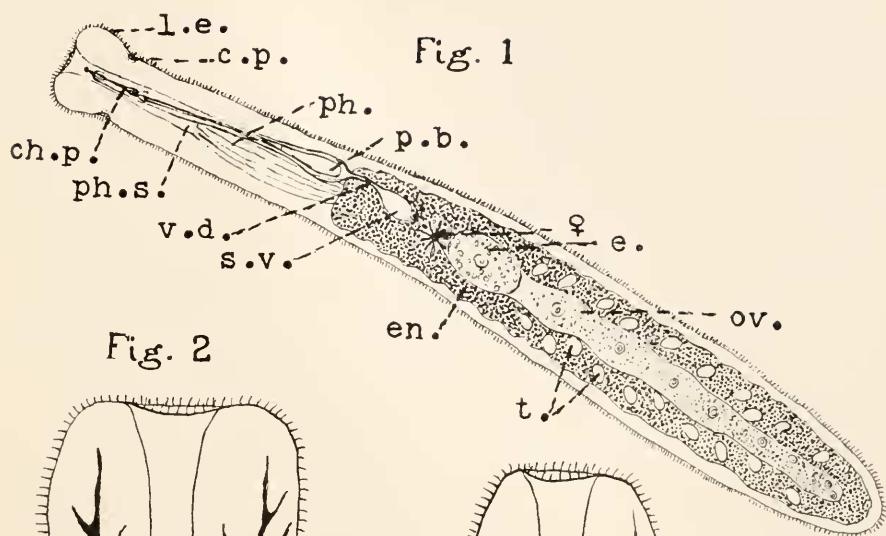


Fig. 2

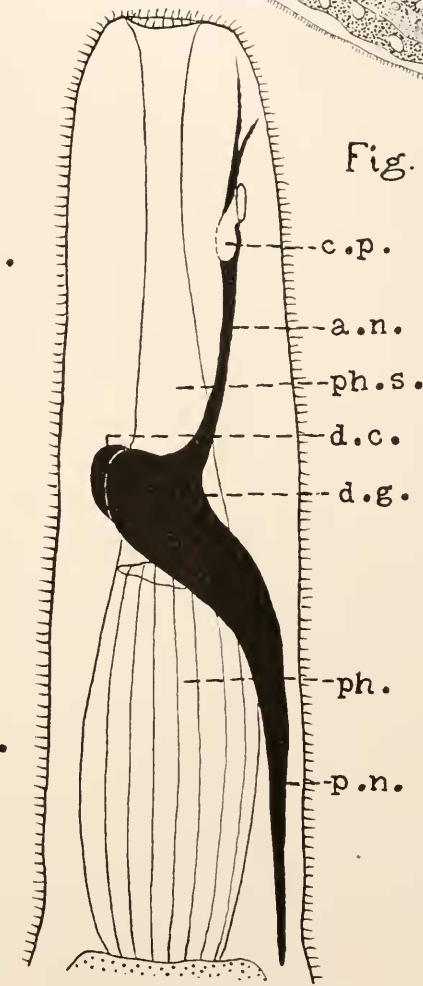
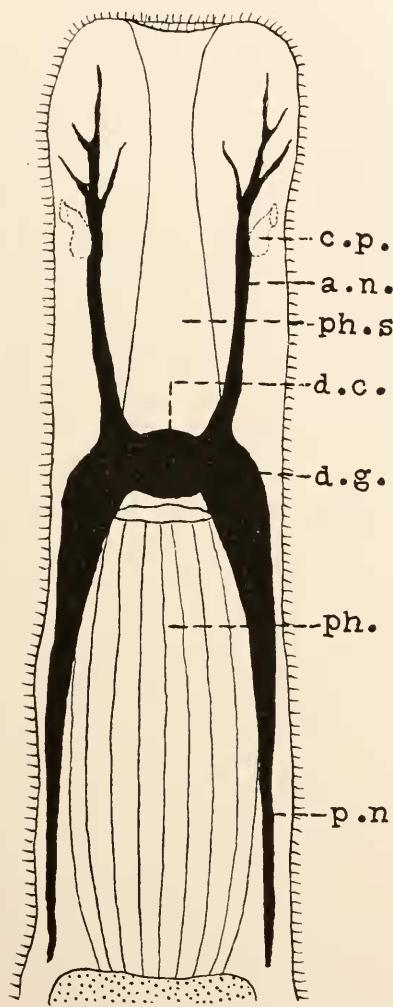


Fig. 3

PLATE II.

FIG. 4. Longitudinal section through a left ciliated pit. General body epithelium (*g.b.e.*); transitory nucleus (*t.*); non-ciliated ridge (*r.*); sensory nucleus (*s.*); pore of gland cell (*p.*); canaliculi (*cn.*); gland cell (*g.*); anterior nerve (*a.n.*); nucleus of gland cell (*g.n.*); lateral muscle fiber (*l.m.*); $\times 1500$.

FIG. 5. Longitudinal section through a left ciliated pit, especially to show the row of four sensory nuclei (*s.*); General body epithelium (*g.b.e.*); anterior nerve (*a.n.*); secretion in pores of gland cell which lead into lumen of pit (*se.*); canaliculi (*cn.*); gland cell (*g.*); nucleus of gland cell (*g.n.*); $\times 1500$.

Fig. 5

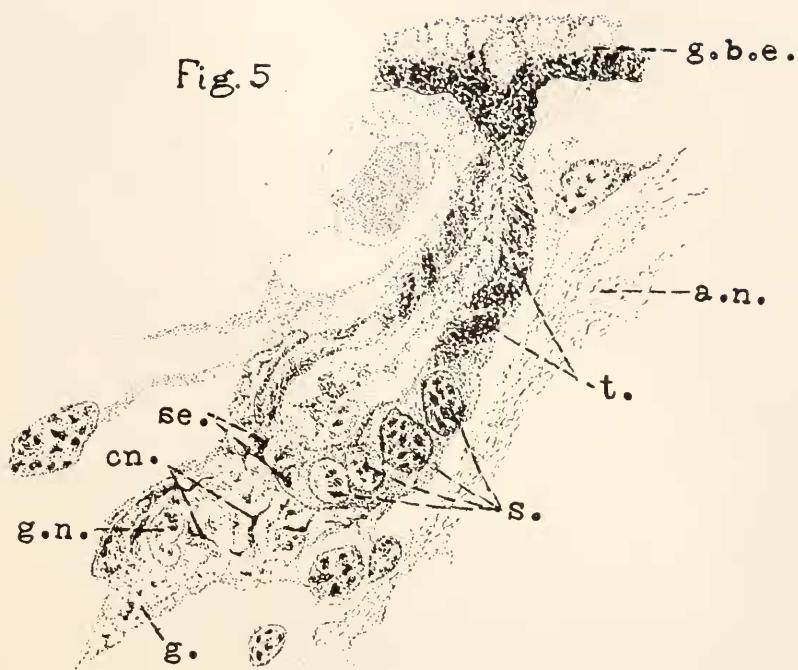


Fig. 4

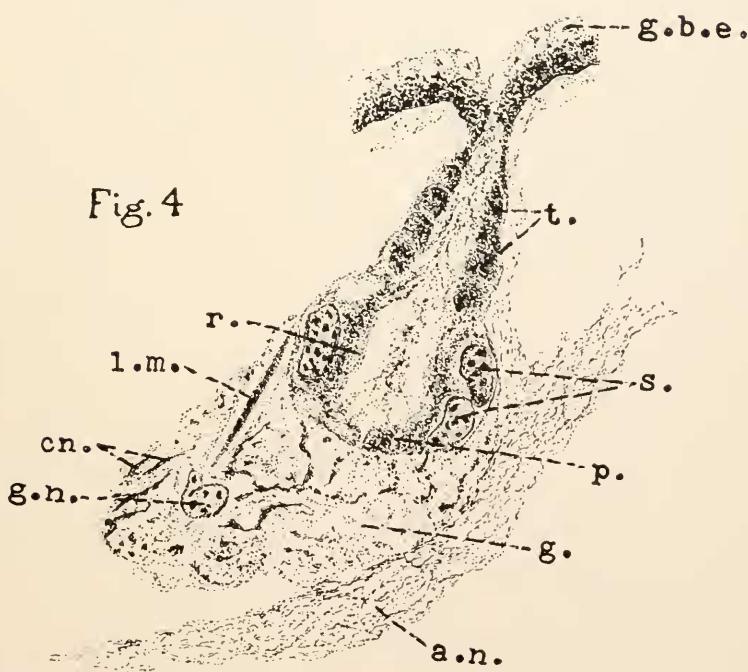




PLATE III.

FIG. 6. Oblique-longitudinal section through right ciliated pit especially to show the three mesial muscle fibers (*m.m.*); associated with the ciliated pits. Sensory nucleus (*s.*); transitory nucleus (*t.*); pores of gland cell (*p.*); canaliculi (*cn.*); gland cell (*g.*); anterior nerve (*a.n.*); non-ciliated ridge (*r.*); $\times 1800$.

FIG. 7. Diagrammatic reconstruction of lateral aspect of right pit. General body epithelium (*g.b.e.*); transitory nuclei (*t.*); mesial application of anterior nerve (*a.n.*); sensory nucleus (*s.*); pores of gland cell (*p.*); emptying into lumen of pit; canaliculi (*cn.*); gland cell (*g.*); bent, ovate nucleus of gland cell (*g.n.*); lateral muscles of wall of pit (*l.m.*); mesial muscles of wall of pit (*m.m.*); $\times 2000$.

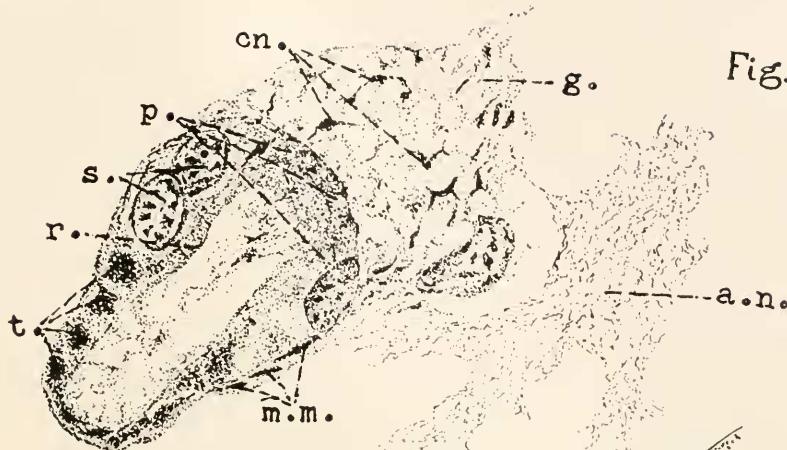


Fig. 6

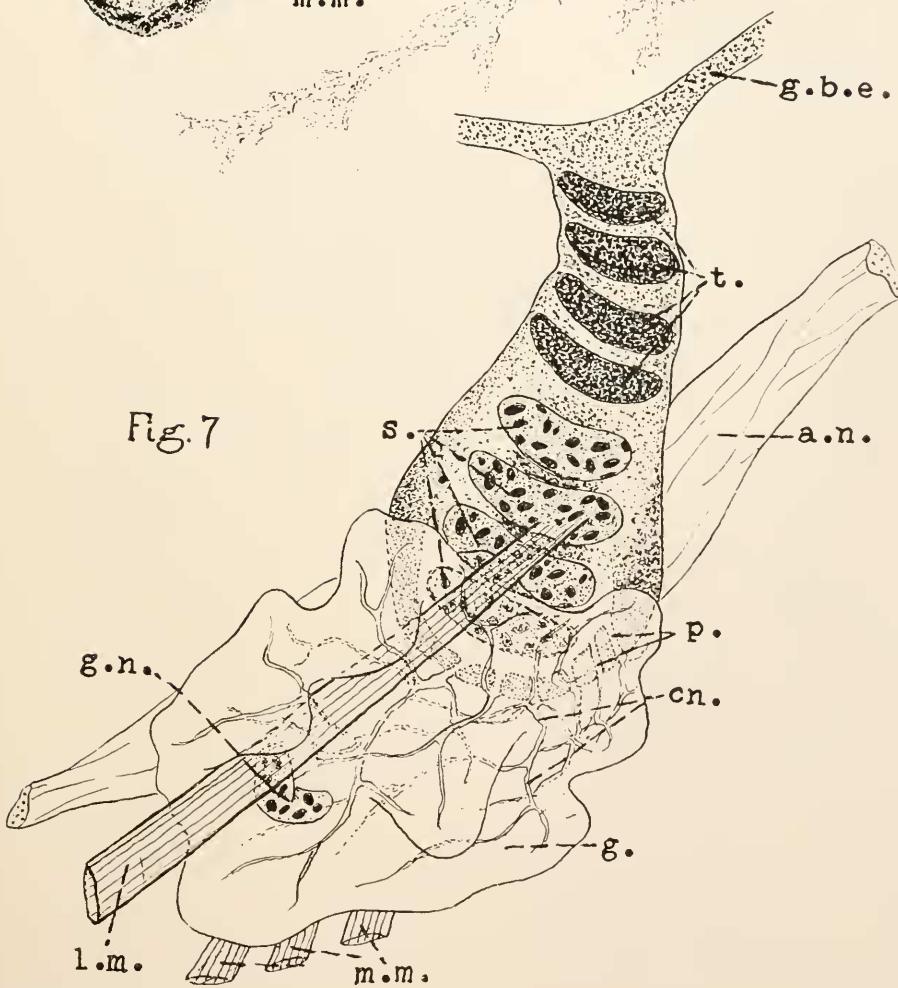


Fig. 7

TESTICULAR ASYMMETRY AND SEX RATIO IN BIRDS.

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It has long been known that in some birds the right testis never attains the size of the left one even at the height of the vernal hypertrophy of the gonads. Newton, in his *Dictionary of Birds* (p. 784) writes that, ". . . generally the left testis is bigger than the right, although both are equally functional." Riddle,¹ working with pigeons at Cold Spring Harbor, found that the discrepancy in size between the two testes in hybrids increased with the width of the cross involved. That is to say, the left testis was proportionately larger in hybrid birds whose parents were of two different genera than in birds resulting from the mating of two congeneric species. The sex ratio of the offspring was found to be apparently interrelated with the discrepancy in size of the testes. Excess of males was always correlated with proportionately larger left testes, while in birds in which the two testes were equal in size the sex ratio was approximately one, *i.e.*, as many of one sex as of the other.

If there is anything in this seeming correlation it should be possible to arrive at some idea of the sex ratio in wild species of birds by examining the testes of adult males in breeding condition. Those in which the left testis is much larger than the right should have an excess of males in their total population and this excess should vary directly with the amount of difference between the two testes of the adult male. The importance of the sex ratio in such matters as the courtship habits and territorial relations of birds must be very great and its accurate determination for any species is prerequisite to a proper understanding of the habits of that species.

With the hope of getting some new light on this subject in wild birds I kept note of the relative size of the testes of all adult

¹ Riddle, Oscar, "Further Observations on the Relative Size of the Right and Left Testis of Pigeons in Health and Disease and as Influenced by Hybridity," *The Anatomical Record*, XIV., 1918, pp. 333-4.

male birds in breeding condition collected during three expeditions to South America, Africa, and the Texan-Mexican border. Only adult males in full breeding condition were of value in this study for in all the birds examined not in breeding condition the testes were invariably equal in size and very small.

In no species was the right testis larger than the left. The two were equal in size in one hundred and four species and the left testis was larger than the right (usually by a very considerable amount) in sixty species. Five species showed both conditions. This is hardly in keeping with Newton's statement quoted above. It should be borne in mind that many of the species are included in the following lists on the basis of the condition found in a single adult male. With further data and the elimination of individual variation the status of some of the species may require modification in this respect.

From my own field notes on all these birds and from all available published accounts of other observers it seems that no general correlation exists between the relative size of the testes of the breeding male and the sex ratio of the species. That is, field observations do not indicate a larger proportion of males in those species exhibiting testicular size assymetry than in those in which the two testes are equal. However it must be admitted that sex ratio is a very difficult thing to determine in the field, particularly as male birds are usually more conspicuous than females and are therefore seen and collected more frequently. In some small groups of species there does seem to be some such correlation as Riddle found in his pigeons. Perhaps the most striking case (and one on which I have sufficient material to eliminate individual variation) is that presented by the Cowbirds.

The Cowbirds are a group comprising three genera and six species. One² of these six species is extremely rare, being represented in the museums of the world by only one adult and three immature specimens and I have no data on it. The condition in the other five is interesting and suggestive. Two of them, *Agelaioides badius* and *Molothrus rufo-axillaris*, have no sexual dimorphism in plumage, are monogamous, and the ratio of the sexes is even, one male to one female. These two species have

² *Tangavius armenti*.

the testes equal in size. The other three *Molothrus bonariensis*, *Molothrus ater*, and *Tangavius xeneus*, have sexual dimorphism in plumage, are more or less promiscuous with a tendency towards polyandry, and the males in all three outnumber the females by at least three to two, or by not less than fifty per cent. In these three species the left testis is much larger than the right. It is interesting to note that of the five the first two (with equal testes in breeding adult males) are the most primitive species of Cowbirds in all respects. *Agelaioides badius* is more or less normal in its reproductive habits but all the others are parasitic, *i.e.*, have no nests of their own and lay their eggs in nests of other birds and leave them to be cared for by these foster-parents. The development of this habit allowed for an increase of males in proportion to females as true mating and pairing were probably of less importance to a parasitic species than to one tied down by nesting and parental obligations. The parasitic habit is simplest in *Molothrus rufoaxillaris* and this species still pairs off in its ancestral monogamous fashion as does the most primitive, non-parasitic *Agelaioides badius*.

In most birds studied however no correlation between testicular asymmetry and sex ratio can be made out. Many more data are needed and I hope that bird collectors will make a point of noting the relative size of the testes in all breeding adult males.

BIRDS IN WHICH THE TWO TESTES ARE EQUAL IN BREEDING ADULT MALES.

Family Ciconiidæ.

Euxenura galatea (Molina).

Family Tinamidæ.

Nothura maculosa maculosa (Temminck).

Family Rallidæ.

Gallinula chloropus brachyptera (Brehm).

Family Charadriidæ.

Charadrius pecuarius pecuarius Temminck.

*Family Jacanidæ.**Jacana jacana* (Linneus).*Family Lariidæ.**Sterna maxima* Boddært.*Geochelidon nilotica* (Gmelin).*Family Cuculidæ.**Crotophaga ani* Linneus.*Guira guira* (Gmelin).*Tapera naevia chochi* (Vieillot).*Lampronotus klaasi* (Stephens).*Clamator cafer* (Lichtenstein).*Coccyzus melocoryphus* (Vieillot).*Coccyzus americanus americanus* (Linneus).*Coccyzus erythrophthalmus* (Wilson).*Family Coraciidæ.**Coracias caudatus caudatus* Linneus.*Family Bucerotidæ.**Lophoceros erythrorhynchus erythrorhynchus* (Temminck).*Family Trogonidæ.**Apaloderma narina narina* (Stephens).*Family Trochilidæ.**Chlorostilbon aureoventris egregius* Heine.*Heliomaster furcifer* (Shaw).*Family Capitonidæ.**Trachyphonus erythrocephala erythrocephala* Cabanis.*Family Indicatoridæ.**Indicator indicator* Gmelin.*Indicator minor teitensis* Neumann.*Family Picidæ.**Picumnus cirratus pilcomayensis* Hargitt.*Campetherina nubica pallida* (Sharpe).*Trichopicus cactorum* (d'Orbigny).

Family Furnariidæ.

Coryphistera alaudina alaudina Burmeister.
Phacellodomus rufifrons sincipitalis Cabanis.
Synallaxis superciliosa Cabanis.
Synallaxis albescens albescens Temminck.

Family Tyrannidæ.

Tæniopætra irupero (Vieillot).
Fluvicola albiventer (Spix).
Euscarthmornis margaritaceiventer margaritaceiventer (d'Orbigny et Lafresnaye).
Elænia albiceps albiceps (d'Orbigny et Lafresnaye).
Empidagra suiriri (Vieillot).
Myiodynastes solitarius (Vieillot).
Pitangus sulphuratus bolivianus (Lafresnaye).
Tyrannus melancholicus melancholicus Vieillot.
Muscivora tyrannus (Linneus).

Family Alaudidæ.

Mirafra sabota Smith.
Mirafra africana transvaalensis (Hartert).
Eremopteryx leucompareia Fischer et Reichenow.

Family Motacillidæ.

Macronyx aurantiigula Reichenow.
Macronyx capensis (Linneus).
Anthus cafer (Sundevall).
Anthus leucophrys (Vieillot).

Family Timeliidæ.

Argya rubiginosa saturata Sharpe.

Family Pycnonotidæ.

Pycnonotus tricolor minor Heuglin.

Family Muscicapidæ.

Batis capensis (Linneus).
Alseonax adustus adustus (Boie).
Melanornis ater pammelaina (Stanley).
Elminia longicauda teresita Antinori.

*Family Turdidæ.**Planesticus amaurochalinus* (Cabanis).*Family Sylviidæ.**Erythropygia pæna pæna* (A. Smith).*Sylvietta whytii loringi* Mearns.*Cisticola semifasciata* Reichenow.*Cisticola chiniana* (A. Smith).*Polioptila cærulea cærulea* (Linneus).*Polioptila dumicola* (Vieillot).*Family Troglodytidæ.**Troglodytes musculus rex* (Berlepsch et Leverkuhn).*Family Dicruridæ.**Dicrurus adsimilis adsimilis* (Bechst.).*Family Laniidæ.**Eurocephalus rueppelli erlangeri* Zedlitz.*Family Prionopidæ.**Prionops poliocephala talacoma* A. Smith.*Family Vireonidæ.**Vireo chivi chivi* (Vieillot).*Vireosylva olivacea* (Linneus).*Family Paridæ.**Anthoscopus musculus* (Hartlaub).*Family Oriolidæ.**Oriolus larvatus larvatus* Lichtenstein.*Family Sturnidæ.**Amydrus morio morio* (Linneus).*Spreo superbus* (Ruppell).*Family Eulabetidæ.**Cinnyricinclus verreauxi* (Finsch et Hartlaub).*Lamprocolius phænicopterus bispecularis* (Stickland).

Family Buphagidae.

Buphagus erythrorhynchus (Stanley).

Family Zosteropidae.

Zosterops virens Sundeval.

Family Nectariniidae.

Nectarinia nectarinoides (Richmond).

Family Compsothlypidae.

Dendroica aestiva aestiva (Gmelin).

Geothlypis aequinoctialis cucullata (Latham).

Setophaga ruticilla (Linneus).

Family Tanagridae.

Thraupis bonariensis (Gmelin).

Family Ploceidae.

Hyphantornis capensis olivaceus (Hahn).

Hyphantornis rubiginosus (Ruppell).

Hyphantornis nigriceps nigriceps Layard.

Hyphantornis jacksoni (Shelley).

Hyphantornis vitellinus uluensis Neumann.

Sycobrotus gregalis (Lichtenstein).

Euplectes xanthomelas Ruppell.

Pyromelana diademata (Finsch et Reichenow).

Pytelia melba melba (Linneus).

Granatina granatina (Linneus).

Sporopipes squamifrons squamifrons (A. Smith).

Dinemellia dinemelli (Ruppell).

Philetairus socius socius (Latham).

Tetrænura regia (Linneus).

Family Bubalornithidae.

Bubalornis niger intermedius (Cabanis).

Family Icteridae.

Agelaioides badius badius (Vieillot).

Molothrus rufo-axillaris Cassin.

Icterus cucullatus sennetti Ridgway.

Family Fringillidæ.

Embernagra olivacea olivascens (d'Orbigny et Lafresnaye).
Saltator cærulescens cærulescens Vieillot.
Saltatricula multicolor (Burmeister).
Serinus mozambicus mozambicus (Linneus).
Sicalis pelzelni Sclater.
Sporophila lineola (Linneus).
Brachyspiza capensis argentina Todd.
Melospiza melodia melodia (Wilson).

BIRDS IN WHICH THE LEFT TESTIS IS LARGER THAN THE RIGHT.

Family Ardeidæ.

Butorides striatus cyanurus (Vieillot).

Family Palamedeidæ.

Chauna torquata (Oken).

Family Phasianidæ.

Francolinus coqui coqui (Smith).

Family Rallidæ.

Limnocorax flavirostra (Swainson).

Family Charadriidæ.

Charadrius collaris Vieillot.
Hoplopterus armatus (Burch.).

Family Laridæ.

Sterna superciliaris Vieillot.

Family Cuculidæ.

Cuculus clamosus jacksoni Sharpe.
Cuculus solitarius Stephens.
Clamator serratus (Sparrmann).
Chrysococcyx cupreus intermedius Hartlaub.
Centropus superciliosus furvus Friedmann.

Family Alcedinidæ.

Halcyon chelicuti chelicuti (Stanley).
Halcyon albiventris albiventris (Scop.).

Family Caprimulgidæ.

Setopagis parvulus (Gould).

Family Coliidæ.

Colius striatus minor Cabanis.

Family Indicatoridæ.

Prodotiscus regulus regulus Sundeval.

Family Phæniculidæ.

Phæniculus purpureus marwitzii (Reichenow).

Family Bucconidæ.

Nystalus maculatus striatpectus (Sclater).

Family Furnariidæ.

Furnarius rufus rufus (Gmelin).

Family Tyrannidæ.

Machetornis rixosa rixosa (Vieillot).

Pyrocephalus rubineus rubineus (Boddært).

Knipolegus aterrimus aterrimus Kaup.

Family Phytotomidæ.

Phytotoma rutila rutila Vieillot.

Family Alaudidæ.

Mirafra fischeri fischeri Reichenow.

Family Muscicapidæ.

Tchitrea viridis perspicillata Swainson.

Lioptilus nigricapillus (Vieillot).

Family Mimidæ.

Mimus saturninus modulator (Gould).

Family Campephagidæ.

Grauculus cæsia cæsia (Lichtenstein).

Family Laniidæ.

Chlorophoneus sulphureopectus suahelicus Neumann.
Laniarius funebris degener Hilgert.

Family Nectariniidæ.

Cinnyris mariquensis mariquensis Smith.
Cinnyris obscura neglecta Neumann.
Cinnyris chalybeus (Linneus).
Cinnyris amethystina amethystina (Shaw).

Family Ploceidæ.

Hyphantornis spilonotus (Vig.).
Melanopteryx nigerrima (Vieillot).
Nigrita schistacea Sharpe.
Anaplectes rubriceps (Sundeval).
Anomalospiza imberbis Cabanis.
Coliuspasser ardens (Boddært).
Coliuspasser eques Hartlaub.
Coliuspasser albonotatus (Cassin).
Diatroþura procne (Boddært).
Urobrachya axillaris (A. Smith).
Pyromelana orix orix (Linneus).
Linura fischeri (Reichenow).

Family Icteridæ.

Agelaius phœniceus phœniceus (Linneus).
Molothrus ater ater (Boddært).
Molothrus ater obscurus (Gmelin).
Molothrus bonariensis bonariensis (Gmelin).
Tangarius æneus involucratus Lesson.
Quiscalus quiscula æneus (Ridgway).
Leistes superciliaris petilus Bangs.

Family Fringillidæ.

Passer domesticus (Linneus).
Passer melanurus (P. L. S. Muller).
Serinus canicollis thompsonæ Roberts.
Poliospiza gularis gularis (A. Smith).
Paroaria cristata (Boddært).
Emberiza flaviventris Stephens.

VARIABLE SPECIES.

Family Cuculidæ.

Cuculus clamosus clamosus Latham.

Lampronotus caprius (Boddært).

Family Dendrocolaptidæ.

Lepidocolaptes angustirostris angustirostris (Vieillot).

Family Ploceidæ.

Vidua macroura (Vroeg.).

Hypochera orientalis Reichenow.

THE FUNCTION OF THE CONTRACTILE VACUOLE IN
PARAMECIUM CAUDATUM; WITH SPECIAL
REFERENCE TO THE EXCRETION OF
NITROGENOUS COMPOUNDS.¹

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INTRODUCTION.

Ehrenberg (1838) was probably the first to consider the function of the contractile vacuole. He asserted that it is a spermatic gland; but no evidence has been found in support of this view.

Lieberkühn (1836), Claparède (1834), Lachmann (1856), Siebold and Stannius (1854), and Pritchard (1861) held that it is a rudimentary heart, which, by its pulsations, produces the circulation of a body-fluid throughout the organism. The demonstration by Jennings (1904) that the vacuole communicates directly with the exterior and discharges its contents into the surrounding medium definitely eliminates such an explanation.

Haeckel (see Kent, 1880, p. 69), Maupas (1863), Bütschli (1887-89), Ehrmann (1894), and others contend that it is a respiratory organelle, or a mechanism for the removal of some of the end products of oxidation; but insufficient evidence has been found to warrant the acceptance of this view.

Stein and Schmidt (see Kent, 1880, p. 69), Griffiths (1888), Calkins (1909), Khainsky (1910), Woodruff (1911), Minchin (1912), Howland (1924), Nowikoff (1908), Shumway (1917), Riddle and Torrey (1923), Flather (1919), and Marshall (1921) believed the vacuole to be an excretory organelle. This view

¹ This paper is a portion of an essay submitted to the Board of University Studies of the Johns Hopkins University in partial fulfillment of the requirements for the degree of Master of Arts. The writer wishes to acknowledge his indebtedness to Professor S. O. Mast, who suggested the problem, and under whose personal supervision the work was done; and to Professor E. K. Marshall of the Department of Physiology of the Johns Hopkins Medical School who offered many helpful suggestions in connection with the literature on the enzyme, urease, and the xanthylol test for urea.

is widely accepted, and is supported to some extent by experimental evidence. In certain instances, the term excretion is limited to mean only the expulsion of a fluid from the cell, as in Carter's (1861) observations. Generally, however, this theory assigns to the vacuole the function of a renal organ of some kind.

Hartog (1888), Calkins (1901), Zülzer (1910), Doflein (1911), and others maintain that the vacuole is an organelle for regulating the hydrostatic pressure within the cell, or a mechanism for removing the excess water which is taken into the body in feeding and through the cell membrane by osmosis. Stempell (1914) constructed a mechanical system which shows clearly that osmosis can be made the causal agent for producing intermittent discharge of a fluid from such a system, but this system was doubtless not intended to be compared with the vacuole in any respect other than the pulsating effect.

The literature concerned with the function of the contractile vacuole consequently reveals no conclusive evidence in support of one theory to the exclusion of all others. The consensus of the evidence, however, seems to indicate that the vacuole is an organelle concerned either with the removal of waste products of metabolism, or with the removal of excess water which accumulates in the organism as a result of endosmosis and feeding. The experiments described in the following pages have a direct bearing on the question as to which of these obtains. These experiments consist of attempts to ascertain the nature of the nitrogenous excretion products of metabolism in *Paramecium caudatum*, and whether or not they are excreted through the contractile vacuole.

THE NATURE OF THE NITROGENOUS END PRODUCTS IN *Paramecium*.

The nitrogenous end products of metabolism in organisms vary in their nature according to the type of organism. For example, in man the bulk of the nitrogen is eliminated as urea, considerably less as uric acid and amino acids, and a very small part as free ammonia; while in the birds and reptiles the bulk of the nitrogen is eliminated as uric acid.

In connection with protozoa Griffiths (1888) made the statement, based on his own experiments, that the vacuole performs the function of a kidney, and that its secretions are "capable of yielding microscopic crystals of uric acid." As material for these experiments he used Amœba, Paramecium, and Vorticella, in mass cultures. In some of the experiments a number of amoebæ were placed on a slide and subjected to the murexide test. The development of reddish-purple color indicated the presence of uric acid. In describing these experiments Griffiths says (p. 132): "After the addition of alcohol minute flakes could be distinctly seen floating in the fluid of certain vacuoles. Bearing in mind the murexide reaction, there is every reason to believe that these flakes are nothing more or less than minute crystals of uric acid." These experiments were repeated many times, generally with positive results, indicating the presence of uric acid. At times, however, the vacuole was found not to contain the slightest trace of uric acid.

Howland (1924) repeated these experiments using several specimens of *Centropryxis* and *Amœba verrucosa*. Cells observed in a dark field immediately after the addition of alcohol did not show the crystals of uric acid in the distended vacuoles, nor did cells after the addition of ammonia show the characteristic murexide reactions, either in the vacuoles or in the culture medium immediately surrounding the organisms. These experiments were conducted during a period of five weeks, always with negative results. Paramecia were subjected to the same test, also with negative results. Howland made use of the Benedict blood-filtrate test for uric acid on cultures of paramecia and amoebæ with positive results. The depth of the color developed varied with the age of the cultures, the older ones giving a deeper color. This indicates that uric acid was eliminated in some way by the organisms.

The question then arises: Is nitrogen excreted by protozoa as ammonia, urea, uric acid, or a combination of these substances? In an effort to answer this question the following experiment was conducted. A large number of paramecia were thoroughly washed, placed in spring water that was free from ammonia and urea, left for a time, and removed by filtration. The

filtrate was then tested for ammonia and urea as described below. The possible presence of uric acid was not investigated at this time.

The paramecia were washed as follows: Culture fluid containing the organisms was poured into a long-necked bottle of approximately one liter capacity until it was filled to within about 5 cm. of the top. Then spring-water was added carefully so as to avoid mixing until the bottle was entirely full. Within five or ten minutes under these conditions the paramecia usually aggregated in very great numbers near the surface of the spring-water. When they had thus collected at the top of the bottle, the surface water containing them was removed with a pipette, and more spring-water added. This process was repeated until most of the animals had been taken from the culture fluid, usually three or four times. The bottle was then emptied, and the water containing the paramecia put into it, after which it was filled with spring-water. The organisms were removed as before. The paramecia were thus washed in fresh spring-water three or four times, after which they were usually found to be free from all heavy debris and large bacterial masses. Smaller organisms which were removed from the bottle with the paramecia were separated from the paramecia by further washing on filter paper. The paper used was about 20 cm. in diameter, and was selected so that the pores were small enough to retain the paramecia, but large enough to allow the smaller organisms to pass through. A liter of water, or more if necessary, was used for this part of the washing process.

After the paramecia had been thus washed they were put into a clean glass beaker; then spring-water was added until the number per cubic centimeter was reduced to from 500 to 2,500 individuals. This was ascertained by counting the numbers in several one cubic centimeter portions and averaging the results obtained. The paramecia were allowed to remain in this water for periods of time ranging from eighteen to thirty-six hours, after which they were removed by filtration.

A portion of the filtrate was tested for ammonia by Nesslerization and the rest for urea by methods described below.

In twenty-two of the twenty-five experiments positive tests

were obtained for ammonia by Nesslerization. In the three in which no ammonia was found the paramecia had been in the water for a period of thirty hours or less. In other experiments in which the length of time was thirty-six hours or more, ammonia was invariably found. This indicates that either ammonia was eliminated in such small amounts that more than thirty hours were required for the concentration to rise sufficiently high to be detected by Nesslerization; or that no ammonia was eliminated as such, the positive test being due to that formed from the hydrolysis of some other excretion product. The latter seems the more probable, for, if ammonia was excreted, the length of time necessary for its concentration to rise sufficiently high to be detected should bear an inverse relation to the number of paramecia per unit volume of water. No relation of this kind was found to exist. The ammonia appeared after thirty to thirty-six hours in all the experiments regardless of the number of animals present. The maximum variation, then, in the length of time necessary for ammonia to make its appearance was twenty per cent., while the variation in the number of paramecia in these same experiments was one hundred per cent. or more. The absence of ammonia in three experiments, and its presence in all the others can be explained if it is assumed that there were too few bacteria present in the three to produce hydrolysis of the more complex excretion products, while in the other experiments there were enough bacteria present. That ammonia can be produced in this way was demonstrated by inoculating a dilute solution of urea with culture fluid. After the solution had been allowed to stand for several hours it gave a positive test for ammonia with Nessler's reagent, indicating that hydrolysis had taken place.

Many tests were made of the materials used in these experiments to prevent the possible introduction of errors. The spring-water was tested for ammonia. The sensitivity of Nessler's reagent was ascertained by finding the greatest dilution possible at which a definite indication of ammonia could be obtained. This dilution was found to be approximately one part in two million. The filter paper on which the paramecia were washed was tested for ammonia.

To ascertain whether or not ammonia is present in the fluid of the vacuole, fifteen experiments were conducted in which Nessler's reagent was injected into the organism. The apparatus used in making these injections consisted of the micropipette developed by Taylor (1925) mounted on the micromanipulator developed by Chambers (1922). The process of injection was performed with the paramecium held by surface tension in a hanging drop of water. The cover-glass bearing the organism formed the top of a cell, the front of which was left open to allow the micropipette entrance. The tip of the pipette was bent up at a right angle to the main shaft to facilitate the injection of the organism suspended on the lower surface of the cover-glass.

In twelve of the injections the contents of the pipette were discharged into the vacuole. In three the pipette did not penetrate the vacuole, but discharged its contents into the cytoplasm in the immediate vicinity of the vacuole. In every test the reagent, which is highly caustic, caused the immediate solution of the whole organism with the exception of the nucleus, which remained intact for a short time before it too was dissolved. In the three tests in which the pipette did not penetrate the vacuole, the surrounding cytoplasm was dissolved as before, but the membrane around the vacuole remained intact for a short time. After several seconds the membrane was dissolved, causing the contents of the vacuole to be emptied into the solution of Nessler's reagent in which it was floating. In none of these tests was the characteristic straw color observed which, in the presence of Nessler's reagent, indicates ammonia. It seems, then, that if ammonia is present in any part of the organism its concentration is below the sensitivity of the reagent. All of these experiments seem to indicate then that very little if any of the nitrogen found in the excretion products of *Paramecium* is excreted in the form of ammonia.

The test for urea referred to above was made as follows: Urease, a specific enzyme for urea, hydrolyzing it into ammonia and carbon dioxide, was added to the portion of the filtrate not used for the test for ammonia in each of the twenty-five experiments mentioned. They were then left for several hours, after

which they were tested for ammonia by Nesslerization. Ammonia was found in the filtrate from every experiment, and generally in higher concentrations than it was in the portions to which no urease had been added. This increased ammonia content after hydrolysis may, then, with a reasonable degree of certainty, be interpreted as indicating that urea from some source had been hydrolyzed with the subsequent production of ammonia. The fact that the three filtrates which gave no indication of ammonia before hydrolysis, gave a distinctly positive test for ammonia after hydrolysis, is alone conclusive in so far as the action of urease is known to be limited to the hydrolysis of urea. Since paramecia were the only organisms present in the water in any considerable numbers, the source of this urea must be attributed to them. It therefore seems evident that in *Paramecium* at least some of the nitrogen is excreted in the form of urea.

IS UREA ELIMINATED BY THE CONTRACTILE VACUOLE?

In an effort to answer this question the xanthydrol precipitation test for urea, described by Fosse (1913) and modified to suit conditions of this experiment, was made by injecting the reagent into the vacuoles. The modified reagent consisted of three to five drops of a ten per cent. solution of xanthydrol in methyl alcohol, in 1 cc. glacial acetic acid. This reagent, in the presence of urea, precipitates long, needle-like crystals of di-xanthyl urea which may be easily recognized. The sensitivity of the modified reagent was found by injecting it, with the aid of the apparatus described above, into a drop of a solution of urea of known concentration. The solution of urea used was successively diluted until the urea content was so low that no precipitate could be observed. It was found that one or more parts of urea in twelve thousand could be detected. The process of injecting the reagent into the drop of solution was observed under a microscope.

Considerable annoyance was encountered in attempting to inject the contractile vacuole of *Paramecium* with the xanthydrol reagent in that fumes from the acetic acid in the pipette killed the organism before the injection could be made. This difficulty

was finally overcome by drawing into the pipette a very small amount of paraffine oil after the pipette had been filled with the reagent. The oil is chemically inert under ordinary conditions and served the purpose very well.

The effect of the reagent on the five paramecia successfully injected was quite striking. The animal was fixed immediately. It assumed an almost hyaline appearance with the exception of the nucleus, food granules, and numerous short, thick crystals which are normally found throughout the body. The contractile vacuole disappeared completely. No trace of the characteristic needle-like crystals of di-xanthyl urea, which are precipitated by xanthydrol in the presence of urea, were found either in that part of the organism in which the vacuole is usually situated, or in the liquid surrounding the organism. Some of these observations were made under an apochromatic oil immersion lens system. It seems from this, then, that if urea is present in the fluid of the vacuole its concentration is too low to be detected with the reagent used, that is, one part in 12,000.

Now the question arises as to whether or not all of the urea excreted could be eliminated by the contractile vacuole if the concentration is as low as this. If not, then it is evident that the contractile vacuole does not function specifically in the excretion of nitrogen, and if this is true it is not an excretory organelle in the ordinary sense of the term.

The concentration of urea that should be in the fluid of the vacuole, if all of it is eliminated through it, was ascertained in the following manner. Maupas (1883) found that the vacuoles of *Paramecium aurelia* evacuate a quantity of water equal to the volume of the entire organism in forty-six minutes at twenty-seven degrees. It was assumed in making these calculations that the relative quantity of water evacuated by *Paramecium caudatum* is approximately equal to that evacuated by *Paramecium aurelia* during the same period and at the same temperature. The average volume of *Paramecium caudatum* was assumed to be that of a cylinder 150 microns long and 35 microns in diameter, and the diameter of the vacuole when distended 10 microns.

On the basis of these assumptions and the observations of

Maupas, the volume of water evacuated by a definite number of paramecia in a definite period of time was calculated. From this the concentration of urea that would be in the fluid of the vacuole, if its function is excretory, was computed. The results of these calculations show that the concentration of urea in the fluid of the vacuole would have to be of the order of one part in two or three thousand to eliminate through it the calculated amount of urea.

The reagent injected into the vacuole is, as previously stated, sensitive to one part of urea in twelve thousand. Since there was in these injections no indication of the presence of urea, it is evident that the results of these experiments are in opposition to the theory that the vacuole is an organelle whose function is the removal of the nitrogenous waste products of metabolism, unless it functions specifically in eliminating uric acid, which is not probable. If, then, it is true that the contractile vacuole functions either as an excretory organelle or a mechanism for regulating the hydrostatic pressure within the cell, it is evident that the results support the latter.

SUMMARY

1. The presence of ammonia and urea in *Paramecium* cultures has been demonstrated.
2. Ammonia is due to the hydrolysis of urea, and is not excreted as such.
3. Nitrogen is eliminated in the form of urea.
4. All the urea excreted can not be eliminated through the vacuole.
5. The function of the vacuole is not the elimination of nitrogenous waste products of metabolism, but is probably the regulation of the hydrostatic pressure within the cell.

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THE USE OF NILE BLUE SULFATE AS A VITAL STAIN ON *HYDRA*.

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In connection with some studies on the reconstitution of *Hydra*, it occurred to the writer that if some method could be devised for marking the various regions in an animal and if these markings would persist, the process of reconstitution could be followed and interpreted much more easily and accurately. However, in reviewing the literature there seems to be little or no application of any of the so-called vital dyes to the invertebrates, particularly the Coelenterata.

Various investigators in studying regeneration in *Hydra* following grafting have made use of animals of two different species which differed from each other, among other characteristics, in color. In this way the fate of the graft could be traced. Others have used individuals of the same species which differed from each other in color shade. Both of these procedures are open to criticism in interpretation of results. In the case first mentioned there are undoubtedly present species differences other than color between the animals used, such as differences in the metabolic rate of the animal as an individual and differences in gradient patterns. In the second case in individuals of the same species, color differences seem to be indicative of different physiological conditions. Observations tend to show, for example, that young *Pelmatohydra oligactis* (Schulze) Pallas (*Hydra fusca* L.) are lighter in color than old animals. The same color difference seems to be true of starved animals as compared to fed. From data gathered by various investigators on *Tubularia* and *Planaria*, there are marked physiological differences between young and old, and starved and fed animals with respect to metabolic rate and, consequently, capacity for reconstitution under various conditions. Obviously some method of vital staining would remove the necessity for using such methods of mark-

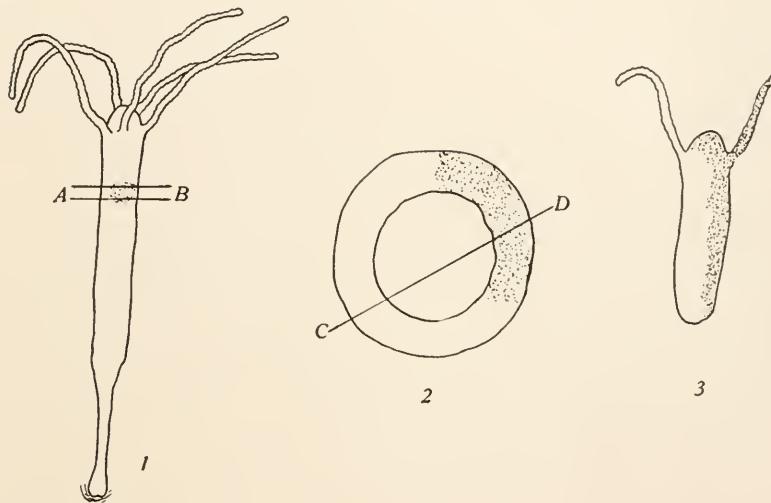
ing as previously described and permit experimentation under more normal, controlled conditions.

Of the various stains considered, nile blue sulfate seemed the most suitable for the work on *Hydra*, particularly on *Pelmatohydra* which is brown in color. The use of nile blue sulfate as a vital stain has been confined for the most part to embryological investigations and applied here for marking the eggs or the embryos. Goodale ('17) used nile blue sulfate in marking the eggs of *Spelerpes bilineatus*, the dye being applied locally in solid form in the least possible amounts by means of a needle. The dye particle was left for a few moments and then removed by washing. A too prolonged application or the application of the dye in excess amount was found to be toxic. Detweiler ('17) used the dye in aqueous solutions of 1:100,000 up to 1:500,000 but concluded that the ratio of 1:150,000 was optimum. In this case the entire animal was stained. Smith ('14) applied an aqueous solution by means of a fine pipette and was able to get some blue spots which persisted. Vogt ('25) uses nile blue sulfate in vital staining by first staining finely divided agar in aqueous solution of the dye (conc. 1:100 to 1:1,000) and after a few days applying in water one of the colored pieces of agar to the tissue to be stained. The color diffuses over after an interval of several hours to one day. However, the stain is not well localized. To localize the dye by this method, the stained agar must be inclosed in glass, tinfoil or paraffin.

After various trials, the method which was found most successful was that of Goodale. The animal, *Pelmatohydra oligactis* (Schulze) Pallas (*Hydra fusca* L.), was placed on a glass slide and the excess water removed from around the animal by means of filter paper. The animal was then transferred on the slide to a dissecting microscope and the smallest possible particle of nile blue sulfate applied in the desired region by means of a needle. Almost immediately the animal was washed from the slide into a stender dish containing water and thoroughly washed in currents of water by means of a pipette. A blue spot was found on the animal at the place of application of the dye. The animal was then sectioned in any way desired and in the following

process of reconstitution of the pieces the blue color was found confined to the original colored cells or the new cells derived from this group as the following example will serve to illustrate.

An animal was stained as described above and a piece removed by two parallel cuts, transverse to the longitudinal axis of the animal (Fig. 1, *ab*). This cross-section, when removed from the animal, appeared as a ring of tissue with the blue spot (stippled area) at one point in the circumference (Fig. 2). This ring of tissue was then divided into two equal portions by a cut (Fig. 2, *cd*) which passed through the blue spot. The two pieces were allowed to reconstitute and at the end of four days two small animals were found similar in marking to Fig. 3.



The process of reconstitution was as follows: the two cut lateral ends of each semicircle had approached and fused, thus forming two small rings similar to that seen in Fig. 2. Further process of reconstitution had then ensued governed by the original polarity of the pieces as is indicated by the color pattern as seen in Fig. 3. Further application of this staining method has been made in investigations not published as yet.

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BIOLOGICAL BULLETIN

A LIPO-GEL REACTION EXERTED BY FOLLICULAR FLUID UPON SPERMATOZOA AND ITS SIGNIFICANCE (LILLIE'S REACTION).

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I. INTRODUCTION.

Some time ago Professor F. R. Lillie observed that when sperm suspensions are mixed with follicular fluid taken from the Graafian follicles a coagulum is obtained (unpublished). As this phenomenon may be significant for the process of fertilization, Professor Lillie suggested that I study the reaction and determine the exact conditions under which it appears. I desire to express my thanks to Professor Lillie for suggesting this subject of research and for placing at my disposal the facilities of the zoölogical laboratories of the University of Chicago.

II. MATERIALS AND METHODS.

Several species were used in the study, chiefly cattle and sheep: Fresh organs obtained at the slaughter-houses of Swift and Company, Chicago,² were always employed. For comparison material was also taken from the goat, opossum, pig, and guinea-pig. The testes and ovaries were usually taken from

¹ From the Hull Zoölogical Laboratory and Whitman Laboratory for Experimental Zoölogy, University of Chicago.

² The author wishes to show his thanks to the officers of Swift and Co., who provided the material necessary for this work.

the bodies of animals just killed and carried to the laboratory in a thermos bottle. Under these conditions the organs remained at a temperature of 25°-30° C. until they were used. The experiments were almost always completed within two to four hours after the removal of the organs from the animals. To establish certain points which required absolutely fresh material, the same series of experiments was performed twice, once at the slaughter-house, within a quarter of an hour after killing, and again in the laboratory.

The spermatozoa were generally removed from different parts of the epididymis by transverse cuts through that structure. Sometimes they were also taken from the vas deferens and from the testes directly and also from the seminal vesicles. The follicular fluid was obtained by pricking the follicles at their most transparent points by means of a fine pipette into which the follicular fluid was drawn by suction.

All organs and especially the female genital tract were taken from the freshly killed animal by myself and examined immediately.

Other details of the technique will be described and explained in connection with the experiments.

III. EXPERIMENTS.

1. When follicular fluid is mixed with spermatozoa taken directly from the epididymis or other parts of the male genital tract a coagulum is obtained in ten to twenty minutes. This coagulum is whitish, semi-opaque, friable, and of a specific gravity greater than that of water; when dissociated with needles, the coagulum breaks up into blocks with sharp edges, indicating a firm consistency. Small fragments of the coagulum when viewed under the microscope appear as homogeneous and transparent masses in which the spermatozoa are imbedded, scattered or in groups. After more complete dissociation of the coagulum into minute fragments one may isolate here and there bundles of fibers coated by a hyaline substance. The surface of the coagulum shines like a fatty surface and does not adhere to water. If therefore the coagulum is set carefully on the surface of water, it will float. When submerged, it sinks, showing that its specific gravity is greater than that of water.

This coagulum is produced at all temperatures between 10° C. and 56° C.; higher temperatures are unfavorable for the reaction. After its formation the coagulum persists at temperatures to 100° C. The fluid around the coagulum produces a second coagulum when heated to 72° C.

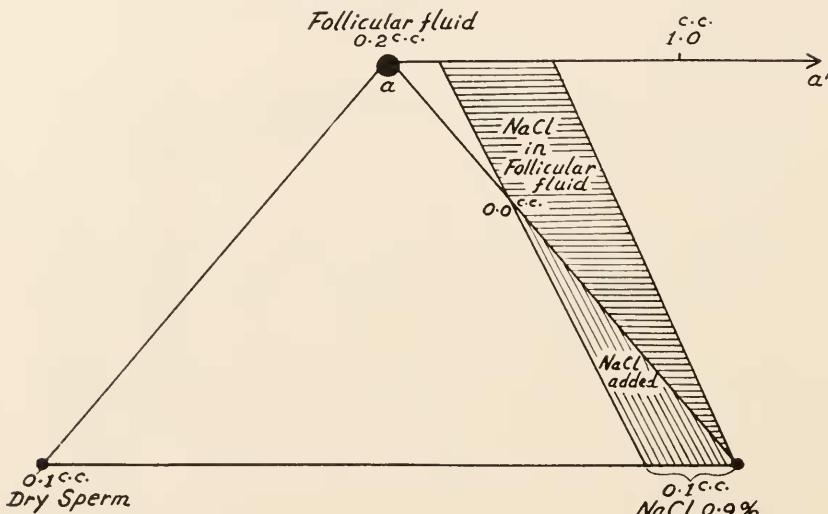
2. When follicular fluid is mixed with spermatozoa only, the results are not uniform. For example in one series of 90 tests, the result was negative in 43 cases, positive in 36 cases, and uncertain in 10 cases. But if the spermatozoa are mixed with 0.9 per cent. sodium chloride solution the reaction is more certainly obtained. For instance in a series of 242 tests, 41 were negative, 198 positive, and 3 uncertain. Furthermore in many cases in which the reaction failed to appear, using follicular fluid and spermatozoa alone, the coagulum was produced upon the addition of 0.9 per cent. sodium chloride solution to the mixture.

This result suggested that the salts which are normally present in the follicular fluid play an important rôle in the reaction. The effect upon the reaction of several salts which commonly occur in organic liquids was thereupon tested. The following salts singly or in mixtures were tried: NaCl , KCl , CaCl_2 , MgCl_2 , LiCl , NaHCO_3 , KHCO_3 , and CaCO_3 . All of these gave a negative result. Only sodium chloride is able to favor and accelerate the reaction.

Since, however, there were a number of negative cases even after the addition of sodium chloride, an explanation for the failure of the reaction in these cases was sought. It was found that the reaction always occurs when the constituents are employed in certain definite quantities. After numerous tests, the following proportions were found to be successful in nearly all cases: 0.9 per cent. sodium chloride, 0.1 cc.; follicular fluid, 0.2 cc.; dry sperm, 0.1 cc. In a series of 128 tests using these proportions only four tests were negative.

The rôle of these proportions in the reaction may be illustrated graphically as in Fig. 1. If too much sodium chloride is added to the same amount of sperm, then the dilution of the sperm is too great and the reaction fails to appear. On the other hand if the quantity of sperm is excessive, then the action of the salt

solution upon the sperm—necessary for the reaction—is inadequate and the coagulum is not formed. With an excess of follicular fluid, the coagulum appears surrounded by a large quantity of fluid; if the quantity of follicular fluid is insufficient, the reaction fails also. From this we must conclude that some substance in the follicular fluid is essential for the formation of the coagulum. A minimum quantity of follicular fluid is thus necessary; this quantity (0.2 cc. to 0.1 cc. dry sperm) is indicated at the point a in the diagram. The line from a to a'



indicates an indefinitely increasing quantity of follicular fluid in which the reaction is still possible, providing the proportions of dry sperm and sodium chloride solution remain constant in the ratio of 1 : 1. We must assume also an active maximum quantity for the sodium chloride solution, namely, a quantity equal to that of the amount of dry sperm. From this amount the proportion of sodium chloride may be decreased indefinitely to zero, at which the reaction is still possible, providing the follicular fluid contains sufficient sodium chloride.

The four negative cases obtained with correct proportions stated require explanation. The following possibilities are suggested:

(a) Possibly the essential substance in the follicular fluid was insufficient in quantity or lacking altogether.

(b) Possibly the quantity of sodium chloride normally present in the follicular fluid was lacking or reduced so that even after the addition of 0.1 cc. sodium chloride solution the amount of this salt present was still insufficient.

(c) Possibly the proportions of the three substances used was not perfectly correct as they were measured by drops usually.

For all practical purposes the proportions of follicular fluid, sperm and sodium chloride solution stated above are sufficiently accurate and seldom fail to yield the reaction.

3. After the conditions necessary for the production of the coagulum had been determined, a new series of experiments was undertaken to establish more definitely the nature of the reaction. The results may be summarized briefly.

(a) The reaction is not produced by other liquids of the organism. I added follicular fluid or dry sperm to the following body fluids: amniotic fluid, blood taken from the jugular vein, defibrinated blood, smear of the mucous membrane of the uterus and tube, urine, cerebrospinal fluid, aqueous humor of the eye, vitreous body of the eye, peritoneal fluid, and pericardial fluid. In all cases the result was negative.

(b) The reaction is interspecific. The following combinations were tried:

Dry Sperm.	Follicular Fluid.
ram	× cow
bull	× sheep
goat	× cow
goat	× sheep
opossum	× cow
guinea-pig	× cow
ram	× pig

In all of these tests the result was positive.

(c) Materials from different sources. Follicular fluid taken from one follicle of the cow was tested with sperm taken from the epididymes of ten different bulls. The result was positive in all cases. Follicular fluid from ten different ovaries of the cow was tried with dry sperm taken from the same epididymis of a bull with positive results in seven cases only. Probably

some of the follicles had not reached the proper stage of concentration of the necessary constituents. However, follicular fluid taken from follicles of ten different sizes (cow and sheep), from the smallest to the largest gave a positive result in all cases. The age of the follicle does not appear to be of importance for the reaction.

(d) Time of reaction. In a series of ten tests (cow and sheep) observed after ten minutes, six were positive and four were negative. In ten tests observed after twenty minutes, all showed the coagulum. In a third series of ten tests, five of which were observed after one hour, five after 24 hours, all were positive. Thus the minimum average time necessary for the reaction lies between ten and twenty minutes. But the reaction is often observable after one minute. When dry sperm is mixed with follicular fluid on a slide, the reaction appears instantly. A delicate layer of coagulum is formed on the surface of the fluid.

(e) Variations in the time of addition of one of the components. In these experiments two of the three components were mixed and the third constituent added after a time interval, varying from one to twenty minutes. The following are the possible combinations:

- (1) Follicular fluid mixed with salt solution; sperm added later.
- (2) Sperm mixed with salt solution; follicular fluid added later.
- (3) Sperm and follicular fluid mixed; salt solution added later.

No noticeable difference in the time of appearance of coagulum was noted in all three cases counting from the time of addition of the third constituent. In the third case the beneficial effect of salt solution on the reaction was again verified.

(f) Variations of temperature.

Dry sperm and follicular fluid were brought to various temperature and mixed (with the addition of salt solution as usual).

The results are summarized on opposite page.

These results show that there is in the follicular fluid a thermolabile substance which is responsible for the reaction. If the follicular fluid is heated to 56° C. the reaction fails. If the sperm is heated to a high temperature, even to 100° C. the reaction is obtained as long as follicular fluid is not heated above 55° C.

Follicular Fluid.	Result.	Dry Sperm.
10° C.....	Positive	10° C.
28° C.....	"	28° C.
37° C.....	"	37° C.
40° C.....	"	56° C.
45° C.....	"	57° C.
50° C.....	"	56° C.
54° C.....	"	56° C.
55° C.....	"	56° C.
56° C.....	Negative	56° C.
28° C.....	Positive	56° C.
28° C.....	"	72° C.
56° C.....	Negative	100° C.
56° C.....	"	28° C.

(g) Freshness of material. Dry sperm and ovaries were kept in the refrigerator and used for the test at various intervals. It was found that sperm fifteen days old and follicular fluid eight days old still yield a positive reaction either when tested with each other or with fresh sperm or follicular fluid respectively. The age of the materials therefore within the limits stated does not affect the reaction.

4. In order to obtain a better understanding of the nature of the reaction follicular fluid and dry sperm have been examined microscopically and the literature has been consulted as to the possible constituents present in these two fluids. I found that dry sperm may contain spermatozoa, spermatocytes, blood, and lipoid substances. Follicular fluid contains or may contain blood (red and white corpuscles and many substances in solution or suspension), granulosa cells, proteins, sodium chloride and other salts, and pigment. I attempted to determine which of these constituents are involved in the reaction.

(a) Blood. It is very difficult to avoid the admixture of blood into either sperm or follicular fluid owing to the cutting or pricking of blood-vessels in obtaining these substances. In my earlier experiments blood was constantly present in my tests and I therefore was inclined to ascribe the reaction to the presence

of blood. Later, however, I took very minute precautions¹ to avoid admixture of blood and found that the reaction was always obtained in the complete absence of blood. I also while at the slaughter-house repeatedly mixed blood from the jugular vein with follicular fluid and never obtained the reaction.² It thus appears that the blood, and by implication the various constituents of the blood are not involved in the reaction.

(b) Proteins. A series of experiments performed with egg albumin gave completely negative results. This result would be expected from the negative finding with blood. Another proof that proteins are not involved may be derived from the fact that the reaction fails at a temperature of 56° C., although the fluid so heated and incapable of producing the reaction still contains proteins which do not coagulate until a temperature of 72° C. is reached.

(c) Sodium chloride. This salt and salts in general never produce the coagulum when mixed with either spermatozoa alone or follicular fluid alone.

(d) Granulosa cells. Follicular fluid was centrifuged and the clear fluid filtered to remove all cells. The absence of cells was verified by microscopic examination. The clear fluid was then violently agitated with glass granules for fifteen minutes with the idea of possibly altering the degree of dispersion of colloidal material that might be present. The fluid was then filtered through four sheets of soft filter paper. The fluid so treated still produces the reaction, indicating that cells or other formed constituents are not responsible for the coagulum. It may be mentioned here that filtered follicular fluid was always employed in all experiments.

(e) Spermatocytes. I was unable to separate the spermatocytes from the spermatozoa and therefore could not test their effect on the reaction separately. But I used material taken

¹ Ovaries taken directly from the body; washing them very carefully in laboratory before experiments; after that drying by absorbent paper; dissecting the follicles with a sharp knife until they appear like transparent vesicles. Only after that treatment puncture of follicles.

² In this case the anticoagulant effect of the sperm or of follicular fluid can be explained by the properties of NaCl and fatty substances, both able to stop the normal coagulation of the blood. Gilbert et Weinberg, "Traité du sang," 1913-1921, p. 15-16.

directly from the testes of animals of various ages and always obtained a positive reaction. Material from the testis may be supposed to contain a larger percentage of spermatocytes or cells in other stages of spermatogenesis than material taken directly from the epididymis or vas. No difference can be seen in the reaction however.

(f) Spermatozoa. Spermatozoa taken from any part of the epididymis, testis, vas deferens, or seminal vesicle always yield the reaction under the conditions previously described. Spermatozoa have been ground in a mortar for ten minutes, then mixed with salt solution, centrifuged, and filtered through four sheets of filter paper. The fluid obtained in this fashion was observed under the microscope; no spermatozoa or fragments of spermatozoa could be distinguished. Such a fluid however gives the reaction both with untreated follicular fluid and with follicular fluid that has been agitated, centrifuged, and filtered.

Thus all of the experiments to this point show that a coagulum is produced normally by a particular substance present in the follicular fluid, a product of the ovary, when mixed with products of the testes, especially the spermatozoa. The follicular substance is thermolabile and resistant to the action of mechanical factors. The experiments also show that the spermatozoa as formed cells are not essential to the reaction.

Up to this point in the experiments the reaction appeared to be perfectly specific: only products of the ovary act upon products of the testes.

5. Rôle of lipoids. Through certain microscopical observations, however, a new line of experimentation was suggested to me. Whenever spermatozoa treated with sodium chloride or with follicular fluid were studied under the microscope it was noted that after a short time the field of the microscope became filled with numerous small droplets, refractile, perfectly round, of the dimensions of cocci, and engaged in active brownian movements. The droplets increase in number with the degree of concentration of the salt solution.¹ When the suspension begins

¹ This phenomenon of drop production by action of salt solution is very clearly explained in Aschoff, L., "Zur frage der tropfigen Entmischung," *Verh. deutsch. path. Gesell.*, 17, Tagung München, 1914, p. 103-109. The influence of NaCl

to dry, the droplets run together into larger and larger drops which are also refractile and homogeneous. Their appearance is very similar to that of a fatty substance.

These droplets persist in normal hydrochloric acid but dissolve in sodium hydroxide (0.9 per cent.). When treated with a saturated solution of Sudan III. in 96 per cent. alcohol, all of the droplets turn red and appear as bright red points. These facts indicate the lipoid nature of the droplets.

The final analysis of this substance is a task for the chemist. The important fact in connection with these experiments is that the spermatozoa are always accompanied by a variable quantity of a lipoid substance.¹ This lipoid substance is responsible for the reaction with which this paper is concerned. The lipoid is in the state of a colloidal sol. When mixed with salt solution its state of dispersion is gradually changed but it still remains in the sol state. Upon the addition of follicular fluid, the thermolabile substance in this fluid exerts an influence upon the lipoid changed by salt solution and causes it to pass into the gel state. A coagulum is thus formed. Because of these characteristics of the reaction, I have designated the latter as a lipo-gel reaction.

The question to be next considered is whether the lipoid substance which surrounds the spermatozoa is specific for the reaction as is the thermolabile substance present in the follicular fluid. An article of Kölliker's suggested to me that the lipoid accompanying the spermatozoa might be similar in nature to myelin,² found in the central nervous system. I therefore mixed follicular fluid with a cell-free extract obtained by grinding up spinal cord (cow) in salt solution in a mortar and filtering. The solution upon lipoids was observed also by Runnstrom, J., "Weitere Studien über die Veränderungen der Lipoide bei der Befruchtung Seeigeleies," *Arch. Zool.*, 16, 1924, p. 1. The same effect was obtained instead of NaCl solution with thymol by Bidermann, W., "Über Wesen und Bedeutung der Protoplasma lipoide," *Pfluger's Archiv*, Bd. 202, 1924, p. 223.

¹ The same idea of a substance surrounding the spermatozoa is considered by Braus-Redenz and Redenz, H. E., *Nebenhoden u. Samenfäden. Anat. Anz. Erg.* 58, 1924, p. 121-131. But these authors merely postulate that such a substance exists and give no evidence concerning its nature. As regards the rôle of this hypothetical substance, the study of Redenz does not touch the essential meaning of it because the nature of the substance is not explained.

² A. Kölliker, "Physiologische Studien über die Samenflüssigkeit," *Zeitschr. für wiss. Zool.*, Bd. VII., 1856, p. 201.

result was exactly the same as when follicular fluid is mixed with spermatozoa, except that the coagulum was more translucent.

Other organs which contain lipoid substances were then tested in the same way. The following were tried: thyroid, parathyroid, pancreas, adrenal gland, hypophysis, corpus luteum, skeletal muscle, heart, kidney, cerebrum, cerebellum, subcutaneous fat, subperitoneal fat, uterine mucous membrane, stomach, and lung. A positive reaction was obtained with all of these except the last three. The coagulum in all of these cases was quite similar and appeared under the same conditions as the coagulum produced by sperm and follicular fluid. Size, color, and time of formation vary a little with the organ used but the general result is the same.

Numerous experiments of this kind were performed; in each case tests were made in the slaughter-house with organs from freshly killed animals but without salt solution, and again in the laboratory in the manner described above. Sometimes when the reaction failed to occur when the tests were made at the slaughter-house, it succeeded in the laboratory after the addition of sodium chloride. Thus the reaction with different organs and follicular fluid has the same characteristics as the reaction between spermatozoa and follicular fluid.

Furthermore the typical reaction is again obtained upon mixing a commercial lipoid product cephalin (made by Armour and Company) with follicular fluid. Extracts which I have prepared from spermatozoa and medulla oblongata¹ likewise give the coagulum when mixed with follicular fluid, although the coagulum is rather thin. None of the extracts mentioned nor cephalin give any reaction with amniotic fluid, aqueous humor of the eye, or hydatic fluid.

The reaction in all of these cases invariably fails to occur if the follicular fluid is heated to 56° C.

Thus the supposition that the coagulum results from the action of a particular thermolabile substance in the follicular

¹ Spermatozoa or ground nervous tissue were kept 24 hours in 96 alcohol, than 24 hours more in ether; evaporation on water bath. The residue dissolved in salt solution 0.9; centrifugation for elimination of cells themselves. The filtrates used for tests. Cf. Loewe, S., "Zur physikalischen Chemie der Lipoide, I.-IV., *Biochemische Zeitsch.*, Bd. 42, 1912.

fluid on the lipoid material surrounding the spermatozoa is demonstrated to be correct.

6. The question next to be considered is the rôle of sodium chloride in the reaction. It has been shown that sodium chloride accelerates the reaction. Microscopical observation of the action of sodium chloride on spermatozoa indicates that the sodium chloride causes the minute droplets of lipoid to aggregate into larger droplets. We can conclude that the sodium chloride prepares the way for the action of the follicular fluid by producing some change in the colloidal state of the lipoid. Further research would be required to elucidate this point.

7. An additional point has been determined,—namely, that the lipoid substance occurs on the surface of and between the spermatozoa. This is shown by the following experiments:

(a) When spermatozoa are washed once with 0.9 per cent. salt solution and filtered, both the washed spermatozoa and the filtrate produce the reaction in five to ten minutes.

(b) When such spermatozoa are washed a second time and filtered the washed spermatozoa and the filtrate from the second washing yield the reaction only after 24 hours.

(c) The same spermatozoa after being washed a third time do not give the reaction in 24 hours nor does the filtrate from the third washing yield any coagulum in this time.

(d) These spermatozoa after being washed three times were ground in a mortar with salt solution and filtered again. The reaction again failed.

From these experiments it appears clear that the lipoid substance is not contained inside of the spermatozoa but occurs on their surfaces and between them. The question of the rôle of this lipoid for the life of the spermatozoa and in the process of fertilization will be the subject of new researches now under way.

IV. THE PHYSIOLOGICAL SIGNIFICANCE OF LILLIE'S REACTION.

The first point to be considered is whether the reaction is organ specific. The experiments show that only follicular fluid produce the reaction and so it is organ specific in this sense. *Stricto sensu*, the reaction is however not specific for I have

shown that it can be obtained with products from a large number of organs and tissues. But as the reaction occurs in nature it may be said to be specific, for the follicular fluid under natural conditions does not meet any other fluid with which it produces a coagulum except spermatozoa. It has been shown that the reaction does not occur with peritoneal fluid, smear of the mucosa of the genital tract, or urine. The reaction is produced with lipoids but the only lipoid with which the follicular fluid would ordinarily come in contact is that accompanying the spermatozoa.

We may next discuss the possible physiological purpose of this coagulation phenomenon. At first sight the phenomenon appears to be of a paradoxical nature. In order to perform their function of fertilizing the egg the spermatozoa must retain their motility when introduced into the genital tract; yet it appears from my experiments that in the genital tract they meet a fluid, namely, the follicular fluid, which destroys their motility.¹ Without discussing this matter at too great length the following explanations of the utility of the reaction may be suggested.

1. Lillie has shown that the eggs of certain invertebrates contain and produce a substance which he designates as fertilizin which has the property of activating and agglutinating the spermatozoa and is of importance in the fertilization of the egg. If fertilizin is produced by the mammalian egg it would certainly occur in the follicular fluid. I could not determine the place of origin of the substance in the follicular fluid which gives the coagulating reaction with spermatozoa. It might originate from the ovum, or the granulosa cells. My experiments (p. 230) indi-

¹ It is very surprising that in the whole literature one can not find the slightest indication of this phenomenon. A tremendous number of workers have tried the reaction upon spermatozoa of different kind of substances *excepting* the follicular fluid and this seems the most logical substance to be tried. Professor Lillie was the first to try this reaction and thus opened an entirely new field of experimentation. The origin of the whole question concerning the lipo-gel reaction is included in the long series of works published by Professor Frank R. Lillie. Compare, e.g., "Studies in Fertilization. V. Mechanism of Fertilization in *Arbacia*," *The Journ. of Exp. Zoöl.*, Vol. 16, 1914, p. 523; "Studies on Fertilization. V. The Behavior of the Spermatozoa of *Nereis* and *Arbacia* with Special References to Egg-extractives," *The Journ. of Exp. Zoöl.*, 1913, Vol. 14. As a general review of the entire problem in which my work must be integrated see Frank R. Lillie, "Problems of Fertilization," Univ. of Chicago Press. One can find there also a very rich literature concerning the subject.

cate that the granulosa cells (including presumably the ovum) do not contain sufficient of the substance to give the reaction. It was also shown that blood does not yield the reaction. It is therefore necessary to suppose that the substance responsible for the reaction is a modified product of either the blood or the granulosa cells plus the ovum, or of both, and does not exist as such in any of these objects; or that it is a substance which gradually accumulates in the follicular fluid. In favor of the hypothesis that the active substance originates from the cells of the follicle including the egg is the observation that follicular fluid from atretic follicles does not yield the reaction. The ovary of the cow frequently contains follicles filled with a clear colorless liquid; such follicles do not contain any granulosa cells or any ovum. They are readily recognized by the colorless watery appearance of the contained fluid. This fluid however invariably fails to yield the coagulating reaction, eleven cases having been tested.

The active substance in the follicular fluid does not appear to be identical with the fertilizin of Lillie in its properties. For instance, fertilizin is stated to be very resistant to heat, while the follicular substance as shown above loses its coagulating property when heated to 56° C.

2. Whatever may be the rôle played by the coagulating substance in the process of fertilization I believe that it may have another secondary function which does not necessarily exclude the first suggestion. I am inclined to think that the follicular fluid may serve to protect the peritoneum against possible infection by infected spermatozoa.

I have made a number of observations on the life and behavior of spermatozoa in various media and particularly in follicular fluid. From these experiments it appears that the spermatozoa furnish a very good medium for the growth of bacteria. After standing for three or four hours at room temperature sperm suspensions were seen to contain many cocci and bacilli: and after twenty-four hours they were swarming with microorganisms. For this reason spermatozoa live much longer at low than at higher temperatures. Spermatozoa will live for several days in small glass tubes if they are kept in the refrigerator, but only for

24 to 30 hours at room temperature. On two occasions spermatozoa that had been kept in the refrigerator for nine days showed motility when brought into room temperature.¹ Spermatozoa removed and kept under aseptic conditions may remain alive for eight days in the refrigerator and for four or five days at room temperature. Even infected spermatozoa retain their motility for some time and move through the field of the microscope carrying bacteria with them. I have injected spermatozoa from the ram into the excised uterus of a sheep. In two hours the spermatozoa were found to have penetrated into the uterine tube but they were greatly damaged and fragmented and many of them had been phagocitized. Those spermatozoa which were still alive were in a state of agitation and covered with bacteria. It appears that the surface of the spermatozoa is very sticky and that all kinds of particles therefore adhere readily to them.²

The spermatozoa are placed normally in a very infected organ, namely, the vagina. The penis itself bears some species of bacteria and at all events at the moment of copulation many bacteria are introduced from the outside into the vagina. These bacteria are carried up into the uterine tubes by the spermatozoa and constitute a menace for the peritoneum which is well known to be exceedingly sensitive to infection. The agglutination of the spermatozoa by the follicular fluid would hold these bacteria and permit them to be phagocytized more readily, thus protecting the peritoneum from possible infection.

This hypothesis of the protective rôle of the coagulating substance in the follicular fluid does not exclude the possible importance of this fluid in fertilization; also it is not possible, at the present time, to estimate more accurately the practical efficacy of this protective rôle of the coagulating substance.³

¹ The same observation on favorable effect of low temperature upon longevity of spermatozoa has been repeatedly made *e.g.* by Redenz in the paper already mentioned; Cf. also Mettenheimer, M., "Sperma und künstliche Befruchtung bei Mensch und Tier," *Münch. Med. Woch.*, schr. 72, Jahr. g. 1925, p. 977; Mettenheimer, M., *Arch. f. Gynak.*, 1925, Bd. 162, p. 215.

² I found after I finished my work, that the proof that spermatozoa may transport bacteria was already furnished by Rotter, "Wie ascendiert die Gonorrhöe," *Archiv f. Gynäkologie*, Bd. 117, S. 153. This author has proved that spermatozoa can transport *Bacillus subtilis* and *gonococcus*.

³ I am obliged for editing this paper to Dr. Libbie Hyman to whom I express my best thanks.

THE DISTRIBUTION OF SUBSTANCES IN THE SPERMATOZOÖN (*ARBACIA* AND *NEREIS*).

STUDIED BY INTRA VITAM STAINS AND BY STAINS OF LIPOIDS
ACCORDING TO THE METHOD OF SCHUMACHER.

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I. INTRODUCTION.

In a previous paper² I come to the conclusion that the spermatozoa of mammals are surrounded by a layer of a lipoid substance which is responsible for a reaction observed by Prof. Frank R. Lillie, and called by me the *lipo-gel reaction*.

With other methods and in different animal species, I have been able to analyze much more completely the distribution of this lipoid substance in and between spermatozoa. The following description is a record of these new facts.

In a recent paper Josef Schumacher has devised methods for the identification of lipoids, lipoproteids and fats in the tissues and cells by means of combining different strong reagents with the action of stains. One conclusion of his work is that there is a series of stains (above all Victoria blue and fuchsin) which

¹ From the *Marine Biological Laboratory, Woods Hole, Mass.*

² Gregor T. Popa, 1927, "A Lipo-gel Reaction Exerted by Follicular Fluid upon Spermatozoa (Lillie's Reaction) and Its Significance, BIOL. BULL., Vol. 52, p. 223.

have a special affinity for lipoids, giving a salt by combination with the lipoidic acid. After destroying the proteins by different macerating reagents, one can isolate the lipoids and in this case the staining with the mentioned substances is a specific stain.

I shall not review this method in detail. Everyone who needs to use it must read the original paper: Josef Schumacher: "Zur Chemie der Zellfaerbung VIII. Mitteilung. Ueber die Nachweis der Lipoide in Zelle und Gewebe," *Chemie der Zelle und Gewebe, Zeitschr. f. die Probleme der Gaerhung, Atmung u. Vitaminforschung*, Bd. XII., Heft 5, 1926, S. 433.

I have used his methods (see page 247) but most of the observations were made by simpler methods, using stains recommended by him as specific stains, and in addition various *intra vitam* staining methods. In the latter case the staining was done under the cover slip as in the method of Koltzoff "Studien ueber die Gestalt der Zelle," *Arch. f. Zellforschung*, Bd. II., S. 1, 1909.

II. RESULTS.

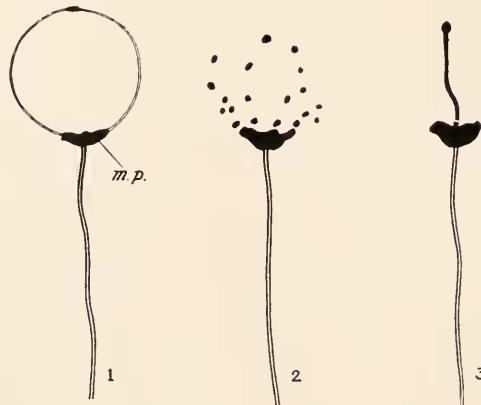
I. THE USE OF STAINS WITH FRESH SPERMATOZOA.

(a) *Victoria Blue and Fuchsin.*

Victoria blue in 1 per cent. solution in distilled water: Stain under the cover slip: one drop of sperm suspension (one drop dry sperm to 10 cc. sea-water) is placed on the slide, and after covering with a cover slip, with a fine pipette one adds at the margin of the cover slip a drop of staining solution. The fluid penetrates by capillarity, advancing more and more slowly. In this way, one gets in the same preparation all degrees of staining, from the strongest color to the complete lack of stain. At the one end of the slide the spermatozoa are overstained, and on the opposite side the spermatozoa are still active.

The first impressive fact is a remarkable change of the shape and size of the spermatozoa. The stain is in distilled water and the spermatozoa are in sea-water: there is a great difference in the osmotic pressure of the two fluids. Thus, a rapid penetration of the staining solution takes place into the spermatozoa, which stain immediately. Shortly after, the heads of the spermatozoa swell, become round and then burst; the following

diagrammatic figures show some of the variations in distribution of the stain (Figs. 1-3).



FIGS. 1, 2, 3. Fresh spermatozoa of *Arbacia* stained with 1 per cent. solution of Victoria blue in distilled water.

When the reaction is too strong, *i.e.*, when the contact of the spermatozoa (in sea-water) with Victoria blue (in distilled water) is immediate, the tails swell also from place to place and one may find shapes as in Figure 4. Frequently, when the reaction is still stronger, the tails twist round the heads and shapes very similar to those described by Koltzoff result (*loc. cit.*, pp. 9-12).

Victoria blue, fuchsin, and the dyes from the same series (Gentian violet, Malachite green, etc.) always stain substances in the acrosome region, in the middle piece, and in the tail. The reaction with Victoria blue, and with the stains in the same class, gives strong reasons for postulating the presence of lipoids in the composition of the acrosome, middle piece, and tail.

Victoria Blue in Alcohol (1 cc. of the distilled water solution in 10 cc. alcohol, 70 per cent.): The solution fixes the spermatozoa at the same time that it stains, and the stain being more dilute, one can see the gradual transformations of the spermatozoa. The heads swell slowly; the tails never swell. The distribution of the stain in the spermatozoa is exactly the same; tip, middle piece, tail. Also, the stain in the region of the acrosome does not always occur, and the middle piece varies in size. By variations of concentration one can have a series of pictures

more and more similar to those obtained by Victoria blue in distilled water.

Fuchsin (1 per cent. distilled water solution): The affinity of the fuchsin for the lipoids is less than that of Victoria blue.

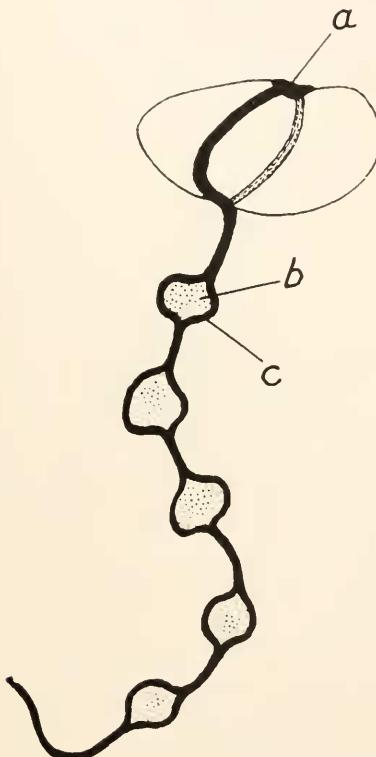


FIG. 4. *Arbacia*. Fresh spermatozoon in 1 per cent. Victoria blue in distilled water—Swelling of tail and twisting around head. *a*, middle piece; *b*, hydrophilic substance; *c*, lipophilic substance.

The staining is accordingly slower and one can follow this process more gradually in the parts of the slide where the effect of distilled water is moderated by a suitable mixing with sea-water.

The new fact obtained by this method is the possibility of observing *changes of shape and variations of size and form of the middle piece*. One can see in its region only granules varying in number (2-6), or a ring varying in size from one spermatozoon

to another, and in the same spermatozoon during the observation¹ until at the end of the reaction almost all spermatozoa show the middle piece as a compact mass, red-stained.

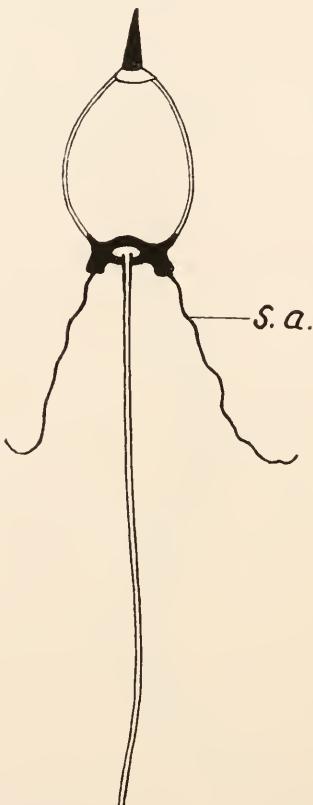


FIG. 5. Spermatozoon of *Nereis* to show sensillæ amoeboidæ.

When the slowly moving spermatozoon shows its tip, one can see that there is really a minute opening. The tip of the spermatozoon is extremely sticky and adheres to everything

¹ The instability of this region was very clearly observed by Retzius and Ballowitz, but they believe that it occurs because of the technique. However, without staining, under conditions approximately normal, the same variations of shape can be noticed. Gustav Retzius, "Die Spermien von *Aurelia aurita* L.," *Biol. Unters.*, N. F., XIV., 1909, S. 67. Gustav Retzius, "Die Spermien der Nereiden," *Biol. Unters.*, N. F., XIV., 1909, S. 69. E. Ballowitz, "Über die Körnige Zusammensetzung des Verbindungsstückes der Samenkörper der Knochenfische," *Arch. J. Zellforschung*, 14 Bd., 1917, p. 355.

which touches it: granules, slide, cover slip, eggs, or to another spermatozoön. Frequently it sticks to the tail of the same spermatozoön.

Victoria blue or *Fuchsin* in sea-water solution: These substances are very feebly soluble in sea-water; but if we keep an excess of stain for several hours in sea-water, a weak solution is obtained in which the spermatozoa can live for ten to thirty minutes and one can see the changes which occur more easily than in distilled water solution.



FIG. 6. Spermatozoon of *Arbacia* showing sensilla amoeboida.

The regions of the spermatozoön already mentioned take the color. But for a short time there is a slight stain on the surface of the head also, before the middle piece is stained. After a variable time the head loses the color gradually and one can see a slow enlargement of the middle piece and the formation of delicate protoplasmic filaments therefrom, which move sporadically. These deserve a more precise description.

The basal part of the head has the appearance of a ring, somewhat thicker than the rest of the head. When the sperma-

tozoön turns in the fluid in such a manner as to show its larger end, one can see very clearly even on fresh preparation, not stained, the presence of this ring. It has different sizes, according to the species (*Arbacia* and *Nereis*) and different outlines on the same spermatozoön. Here and there it is thicker or thinner; sometimes it becomes fragmented in granules and again, it forms a compact mass. In *Nereis* (Fig. 5) there are normally two swellings placed symmetrically, outgrowths of the ring, on which one can distinguish two long filaments as in Fig. 5. In *Arbacia* (Fig. 6), the ring shows in the same way various thickenings (3-5) and these, not so constantly as in *Nereis*, support (usually only one of them) a prolongation ended by a kind of knob.

As the spermatozoön floats in the fluid, these filaments show protean movements, elongation, shortening and thickening, very similar to amoeboid movements. They are constructed of the same material as the basal ring and they are to be considered as prolongations of this. Provisionally they may be called *sensillæ amæboideæ*.¹ In all changes of osmotic pressure, in all media which change or kill the spermatozoa, these sensillæ retract upon the ring where they produce granules projecting a little above the general level.

(b) *Janus Green; Trypan Blue; Neutral Red*
(1 Per Cent. in Sea-water).

These substances are frequently used for staining intra vitam. The penetrating power is different for each. Janus green gave

¹ It is very interesting that the presence of these filaments was noticed by Mischer "Die Spermatozoen einiger Wirbeltiere. Ein Beitrag zur Histochemie," *Verh. der naturf. Gesellsch. in Basel*, 1878, Bd. VI.; the interpretation of them was wrong. E. Ballowitz, in the same species, denies Mischer's observations "Über die Samenkörper des Lachses. Ein weiteres Beitrag zur Kenntnis der Spermien der Salmoniden," *Arch. f. Zellforschung*, 14 Bd., 1917, S. 451; but in an older paper and in other species, "Untersuchungen über die Struktur der Spermatozoen, zugleich ein Beitrag zur Lehre von feineren Bau der Kontraktile Elemente. Die Spermatozoen der Insekten," *Zeitschr. f. wiss. Zoöl.*, 1890, Bd. 50, S. 317, he himself has observed such filaments and even their movements. He even calls them *Wimpelfasern*. The meaning of them is not explained, and he says that they are "rätselhaft." The conditions of observation were bad because he employed always "maceration," after killing the spermatozoa. In fact, the sensillæ are to be observed much more clearly on fresh, slowly motile spermatozoa, without stain.

the best results in our experiments. It penetrates very slowly, and shows excellent contrasts.

By these stains one obtains more clearly the same results as by Victoria blue in sea-water. With Janus green especially, one obtains an entire series of pictures, which prove clearly that *there is a migration of the same substance which stains in Victoria blue*, to the connecting region between head and tail and a large accumulation there of the migrating substance (Fig. 7 a-e). But here and there one can find spermatozoa in which

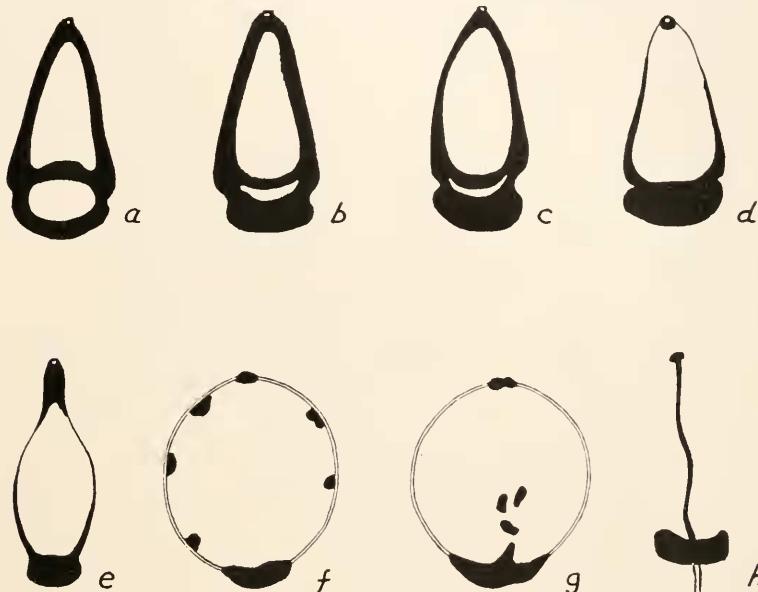


FIG. 7. a-e, sperm heads of *Arbacia* showing stages of staining in Janus green in sea-water; movements of lipochromatic substance. f-h, Victoria blue in sea-water, various conditions. The lipochromatic substance is shown black.

pare the action of Victoria blue and fuchsin in distilled water (Figs. 2, 3, and 4) in which one sees the lipochromatic substance also accumulated on various regions of the head. These facts show the presence in the spermatozoon of *an active cytoplasmic layer*, external to the nucleus, *loaded with lipoids* and *taking* the lipochromatic substance¹ *migrates towards the apex*. Com-

¹ I use this term *lipochromatic substance* for a succinct description, implying only that the substance mentioned takes very strongly the stains reputed by the work of Schumacher to be lipophiles: Victoria blue and fuchsin especially.

various forms. This layer flows in and out of middle piece and forms the sensillæ.¹

2. STAINING METHODS AFTER FIXATION.

Spermatozoa of *Arbacia* and *Nereis* were also studied by the more usual methods of fixing and staining. The fixing methods used were:

1. Corrosive sublimate, saturated solution in distilled water, 100 parts, plus glacial acetic acid, 8 parts.
2. Vapor of osmic acid, 2 per cent. aqueous solution.
3. Flemming, strong solution.
4. Formalin, 10 per cent.
5. Heat, after rapid evaporation.

The following stains were employed:

1. Iron-hæmatoxylin (Heidenhain) and eosin.
2. Iron-hæmatoxylin (Heidenhain) and safranin.
3. Fuchsin.

These procedures applied to the study of the spermatozoa, when united with the observations on living material, give us the conviction that *there is no one fixing fluid which preserves perfectly the form and the structure of the spermatozoa*.² The fastest acting fixing agents (as vapor of osmic acid) and the most penetrating fluids (as acetic acid plus *k*-bichromate) change the size, the configuration of the spermatozoa and the mutual relations of the substances in it. The outlines of the head slightly change, the heads swell, the sensillæ disappear, the ring changes its shape, and the lipochromatic substance migrates.

¹ I have strong reasons to believe that Ballowitz has observed also the migration of the peripheral substance of the spermatozoa, "Untersuch. über die Struktur der Spermatozön der Fische, Amphibien, Reptilien," *Arch. f. mikr. Anat.*, 36 Bd., 1890, S. 225. His explanation is not satisfactory. He believes that the change of color in various parts of the head of the spermatozoön is a consequence of stain diffusion in the medium (p. 239). But the changes can be better observed by the method of Victoria blue, fuchsin and Janus green than by his method; and one can also observe the accumulation of the substance in the connecting region even on spermatozoa not stained at all.

² Students of spermatozoa note the same thing. This fact is demonstrated particularly clearly by C. Pictet, "Rechérches sur la spermatogénèse chez quelques invertébrés de la Méditerranée," *Mitteil a. d. Zoöl. St. Neapel.*, 10 Bd., 1891-93, S. 75.

3. SCHUMACHER'S METHODS.

A.

One makes a smear with sperm as thick as possible, and before the sperm is dried the slide is plunged into HCl solution 1:4. The slides remain in the acid 24 hours. After that time, wash in distilled water and stain for 5 minutes in 1 per cent. Victoria blue in distilled water. Again 5 minutes in distilled water. Mount after usual treatment in cedar oil, or, in order to have clearer pictures (but more unstable) one can mount directly in water and seal with paraffin.

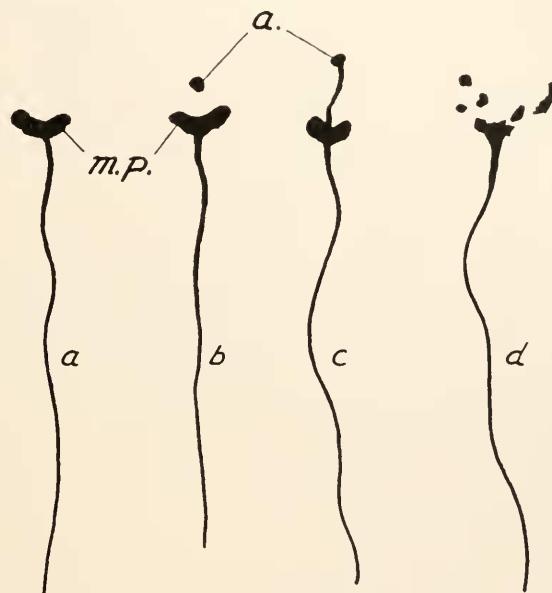


FIG. 8. Spermatozoa of *Arbacia* prepared according to method A of Schumacher and stained in Victoria blue.

The idea of this treatment is as follows: The various chemical components of cells show a different resistance to the various chemical agents. By combining the destructive agents and the time of acting, it is possible to isolate by turn the chemical components of the cell and detect them afterwards.

Schumacher uses strong acids in order to destroy the proteins of the cell. The remainder, after maceration, must be some

substance not influenced by acids, essentially lipoproteins (proteins united with lipoids) and lipoids. If this is true, the stains characteristic for proteins, such as methylene blue, will stain no more, and on the contrary, the stains of the fuchsin series still will give a coloration.

This method was used by Schumacher in studying leucocytes, yeast cells, liver, lung, and skin. I have applied this method in studying the spermatozoa.

With the above method one obtains with Victoria blue the following pictures (Fig. 8); staining with methylene blue fails completely. This demonstrates that the various portions of spermatozoa stained with Victoria blue by the procedures already described, resist the action of strong acids, while the other portions are destroyed. Therefore, according to the theory of Schumacher, we can say that *in the construction of the tail, middle piece, and acrosome of the spermatozoon there is a great quantity of lipoid substance.*

B.

For deciding if in an organic construction, which resists the action of acids and still is stained by Victoria blue, there are free lipoids or lipoids associated with proteins (lipoproteins), Schumacher uses another procedure: Smears made as in A (above) are placed for 24-36 hours into a mixture of alcohol and ether. Afterwards the slides are transferred for 24 hours to HCl 1 : 4. The staining following is identical with that described in A (above).

The idea is this: The alcohol-ether dissolves and washes out *free* lipoids, but does not touch the lipoproteids; afterwards HCl destroys the proteins, and again the lipoproteids remain intact, and thus they are isolated. If, after such a treatment, we still obtain a coloration, we can say that there is a lipoprotein component.

Applied to the study of spermatozoa, this method gives the pictures shown in Fig. 9. A pale reticulum built by interlacing tails, and here and there some knobs at the ends of fibers, inferred to be the remains of middle pieces. It is to be noticed also that the fresh preparations immediately after staining with Victoria

blue, are much clearer than the preparations mounted in balsam, and particularly after treating with xylol.

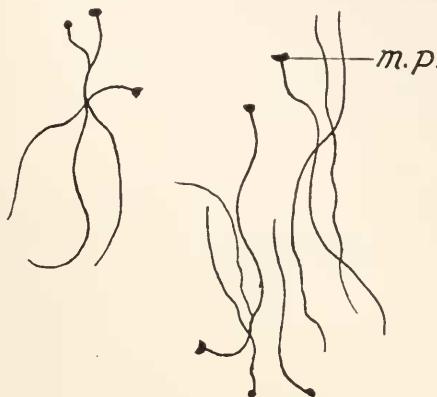


FIG. 9. Spermatozoa of *Arbacia* prepared according to method B of Schumacher and stained in Victoria blue.

Summarizing the facts obtained by the method of Schumacher, one can say that in the structure of the spermatozoön there are free lipoids and lipoproteids which are located normally in the tail, middle piece, and acrosome. The facts described in 1 and 3, connected with the facts observed in living spermatozoa, demonstrate that the lipoids and lipoproteids of the tail are located *on the surface*.

4. OSMIC ACID (1 PER CENT. AQUEOUS SOLUTION 24 HOURS,
OR 2 PER CENT. AQUEOUS SOLUTION FIXING
BY VAPOR 1-5 MINUTES).

Osmic acid shows in *Arbacia* very similar pictures to those obtained by the lipophilic stains. One can see very clearly the basal ring, the tail, the middle piece; the conical part of the head appears darkened and unburnished, which gives us the impression that the substance darkened by osmic acid is located on the surface of the spermatozoön. The substance between spermatozoa is darkened too. On fresh preparations this substance can be distinguished still more clearly. If spermatozoa are shaken in sea-water, and from the clear fluid, which separates in the upper part of the test tube, a drop is taken and submitted

to the action of osmic acid vapor, one sees under the microscope very fine round droplets, black and refractile, evidently originating in the intercellular substance. The preparations by osmic acid give us the impression that there is *on the surface of and between the spermatozoa a substance which contains fat and which surrounds the cells as a mantle.*

5. SUDAN III.

Sudan III. shows in the same way as osmic acid, the presence of fatty globules accumulated between the spermatozoa after treating them with the reagents.

6. THE MACROCHEMICAL TEST WITH VICTORIA BLUE.

If 1 per cent. Victoria blue in distilled water is mixed with ether, one obtains, after shaking, an emulsion which, after 10-30 minutes, separates in two distinct layers: the *non-stained* ether above, and the strong colored Victoria blue below. If to the same mixture one adds dry sperm, after shaking, the ether separates again, but now it carries with it the fatty substances from the spermatozoa. These substances are already stained by Victoria blue, and, therefore, the ether in the upper part of the test tube is blue. If, instead of spermatozoa, we use only the fluid separated after centrifuging a sperm suspension, we may have a complete transfer of color into the ether, the lower layer, representing the water of the Victoria blue solution, being now uncolored. This reaction corroborated by the tests of Schumacher and the reactions with Sudan III. and osmic acid demonstrates that around and between spermatozoa there is a substance rich in unsaturated and neutral fats.

7. OBSERVATIONS ON LIVING SPERMATOZOA IN SEA-WATER.

Observations are made with the oil immersion lens. By varying the focus, the strength of the light and the eye pieces, one can see quite clearly many of the facts obtained by staining methods. Without doubt, the value of this kind of observation is very great, especially when combined with the above mentioned methods; more and more one reaches the conviction that there are in the spermatozoa two different zones: a darker one at the

periphery, which becomes thicker towards the connecting region between head and tail, where it constructs a large ring. The central zone is more refractile. It is larger towards the connecting region and diminishes towards the apex.¹ Almost all transformations of the ring can be seen in fresh spermatozoa; the sensillæ amoeboidæ show most clearly. The migration of substances can be deduced by seeing the changeable sizes of various parts of the *same* spermatozoön during the observation.

In the preceding observations, there is one fact which deserves more emphasis: this is the *stickiness of the pointed part of the head, and of the connecting region too*. Very frequently, and chiefly in some particular reactions, taken into consideration below, the spermatozoa bunch together, sticking by these two regions. By careful and patient observations of fresh spermatozoa, during longer time and in various media, one gets the impression that the spermatozoa eliminate through the point of the head (where really there is an exceedingly minute opening) very small amounts of an extremely sticky substance.

8. THE EFFECTS OF EGG-WATER ON THE SPERMATOZOA.

The mutual relations of the various constituents of the spermatozoön, being established and knowing the lability of these relations, a large field for studying the topographical changes of the principal substances in various media is opened. I shall limit myself here to one point: *the changes which occur in the spermatozoa in egg-water, which might be expected to resemble changes preliminary to fertilization.*

This is the classical phenomenon studied by Lillie.² It is very well known at the present time that there is exerted an agglutinative action upon spermatozoa by the sea-water in which sea-

¹ Mischer (*loc. cit.*) noticed the same thing. Ballowitz in *Untersuch. u. die Struktur d. Sp.* (Fische, Amph. Rept.), p. 249, distinguishes in the head of the spermatozoön two different zones, and he says that in the process of swelling "the internal zone leads." In fact, it alone swells the external substance merely changing its place.

² Frank R. Lillie, "Studies of Fertilization. VI. The Mechanism of Fertilization in *Arbacia*," 1914, *Journ. of Exp. Zoöl.*, Vol. 16, p. 523. Frank R. Lillie, "Studies of Fertilization. V. The Behavior of the Spermatozoa of *Nereis* and *Arbacia* with Special Reference to Egg-extractives," *Journ. of Exp. Zoöl.*, Vol. 14, 1913, p. 515.

urchin eggs have been for a short time. The reaction is instantaneous. The spermatozoa exhibit great activity and bunch together, for a while, in compact clusters of various sizes.

What is the immediate cause of the agglutination residing in the spermatozoa? Is it due to a change in the arrangement of the substances, or to an active elimination of the sticky substance observed in fresh suspensions of spermatozoa?

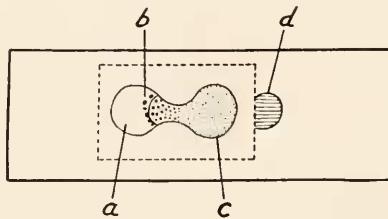


FIG. 10. Arrangement of agglutination experiment; *a*, egg-water. *b*, clusters. *c*, sperm-suspension. *d*, drop of Janus green.

One drop of egg-water and one drop of sperm suspension are placed on a slide (Fig. 10). A very narrow bridge is established between the two drops. Immediately the agglutination takes place where the drops meet, and slowly spreads, so that we have again various stages of a phenomenon on the same slide.

At the left end of the egg-water drop (Fig. 10) the fluid is still clear; at the middle, agglutinated clusters of spermatozoa in great activity; at the right side of the sperm suspension drop, the spermatozoa in various degrees of motion, are in a homogeneous suspension.

In these conditions, one keeps the slides various lengths of time (1-10 minutes). Afterwards one puts very carefully a cover slip with another drop of egg-water underneath (to mitigate the power of capillarity). Many bunches of spermatozoa are spread out, but many other resist, and one can observe, under the oil immersion lens, what happens in the clusters.

A long series of transformations of the individual spermatozoa occur, represented by the next sketch (Fig. 11). These transformations take place under the influence of egg-water only. But in order to see them more clearly, one may add at the margin of the cover slip a drop of 1 per cent. Janus green in

sea-water. The changes shown diagrammatically in Fig. 11 are: (a) A normal spermatozoön; (b) Initial changes in the arrangement of lipochromatic substance; (c) The nucleus swells a little

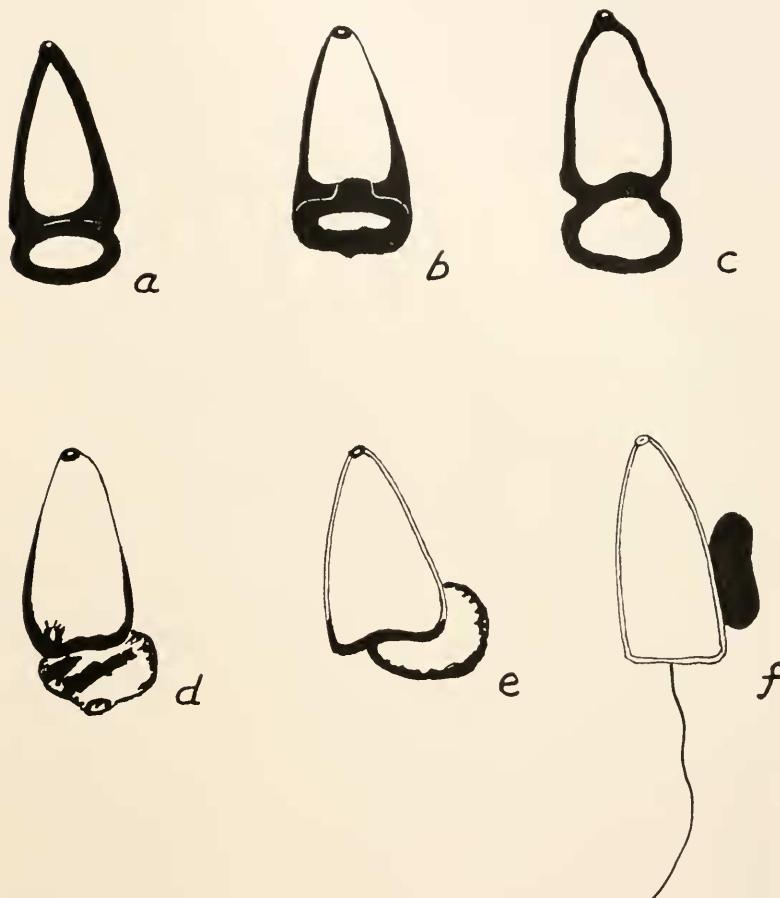


FIG. 11. Behavior of the spermatozoa of *Arbacia* in egg-water; intra-vitam staining with Janus green. See text.

and moves towards the base of the head. At the same time the lipochromatic substance divides into two, a part follows the nucleus, covering it, another part remains in the region of the basal ring. Thus one can see in fresh preparations *on the connecting region* of the spermatozoa two transversal dark zones, between which there is a refractile one. On stained preparations

there are two colored strips and one light band; (d) The same process, much more advanced; (e) The lipochromatic substance accumulated in greater amount, shifts from the connecting region; (f) A round or slightly oval body is thus extruded from the head, and it takes a lateral position, where it persists without any modification, and the whole spermatozoön becomes perfectly quiet. It is to be noticed that the spermatozoa which have extruded this *lateral body* do not swell. On the other hand, the spermatozoa which have not extruded it, swell and become round.

III. DISCUSSION.

Summarizing all the facts revealed by the various methods above described, concerning the topographical distribution of the material in the spermatozoön (in *Nereis* and *Arbacia*) there is to be distinguished a substance stained by lipophilic stains, *the lipochromatic substance*; and another substance very avid for water, *the hydrophilic substance*. The lipochromatic substance is located at the periphery of the spermatozoön, and its thickness is variable in different regions; towards the connecting region it constructs a large ring and the sensillæ; in the tail it takes part in the construction of the tail mantle. This substance is not avid for water, and therefore does not change its volume in various hypotonic solutions. On the other hand, it shifts position readily, and it accumulates normally in the connecting region of the spermatozoön (Fig. 11).

The hydrophilic substance is located in the axis of the spermatozoön, and does not take the lipophilic stains. In the head it includes the nucleus or may be represented exclusively by the nucleus. It is extremely hydrophile, swells in water (hypotonic solutions) and thus enlarges the head of spermatozoön until more than three times diameter. This substance is centrally located in the head and also in the axis of the tail.¹

Apparently the hydrophilic substance of the head is nothing else than the nucleus and the lipochromatic substance must be

¹ Concerning the sensitiveness of these cells to the changes in the osmotic pressure see: Richard Goldschmidt, "Kleine Beobachtungen und Ideen zur Zellenlehre III Die Bedeutung der atypischen Spermatozoen," *Arch. j. Zellforsch.*, 14 Bd., 1921, S. 290. Richard Goldschmidt, "Versuche zur Spermatogenese in vitro," *Arch. f. Zellforsch.*, 14 Bd., 1921, S. 421. Josef Speck, "Neue Beiträge zum Problem der Plasmaprozesse," *Zeitschr. f. Zellen- u. Gewebelehre*, 1 Bd., 1924, S. 278.

the cytoplasmic component of the spermatozoon loaded with lipoids and lipoproteids.

The cell is surrounded by a very thin membrane, the presence of which is obvious when the heads swell and explode in hypotonic solutions. The membrane must be very elastic and permeable to water. The whole complex is protected externally by a thick *layer of fatty substance*, darkened by osmic acid and detected also by Sudan III. and by macrochemical analysis with Victoria blue.¹ Its chemical composition is still not clearly demonstrated.

Mixing dry sperm with sea-water one can clearly see this substance spreading out on the surface film like oil. In the dry sperm under the microscope, one can see how abundant this intercellular substance is. In this medium the spermatozoa live longer than without it. Presumably the contact of the spermatozoa with the water is very much mitigated by the surrounding fatty medium, so that changes of osmotic pressure influence the spermatozoa gradually, and the modifications of form occur very slowly. This layer of fatty substance may be considered on the whole as a *buffer* between a very sensitive structure (spermatozoon) and a salt solution at a very high concentration (sea-water). Certainly the fatty substance may have some other important significance, too, not yet taken into consideration.

As soon as the concentration of the medium is changed and the fatty medium is modified, the spermatozoa react first by motility, and secondly the mutual relations of the two substances (hydrophilic substance → lipochromatic substance) undergo changes. As a result we have the various pictures of the spermatozoon already demonstrated.

For completion of the general sketch of the spermatozoon there is to be remarked the presence at the distal end (that is

¹ This layer outside the spermatozoon has been frequently noticed recently by various authors: Redenz, E., "Versuch einer biologischen Morphologie des Nebenhodens," *Arch. f. Mikr. Anat. u. Entw. Mech.*, 1924, 103 Bd., S. 391. Gellhorn, E., "Physiologische Untersuchungen an Spermatozoen und Eiern. Ein Beitrag zum Befruchtungsproblem. Sammelreferat," *Arch. f. Mikr. Anat. u. Entw. Mech.*, 1924, 101 Bd., S. 437. Redenz, E., "Versuch einer biologischen Morphologie des Nebenhodens. II. Die Bedeutung elektrolytarmer Lösungen für die Bewegung der Spermien," *Wilh. Roux' Arch. f. Entw. mech. d. Org.*, 106 Bd., 1925, S. 290. T. von Lanz, "Über Bau und Funktion des Nebenhodens und seine Abhängigkeit von der Keimdruse," *Zeitschr. f. die ges. Anat. Z. f. Anat. u. Entw. g.*, 80 Bd., *Festschr. f. S. Mollier*, 1926, S. 177.

to say, the tip of the head) of a minute opening, which it is convenient to call by analogy *micropyle*; and also at the connecting region there is another small ring very easily colored by stains which have affinity for chromatin, especially by *carbol-pyronine*. This is the classical proximal centriole.¹

We have seen above the various changes of the mutual relations of the two substances in the head of spermatozoön. The end result of these changes is the elimination of the *lateral body*. This elimination phenomenon occurs, also, under various other circumstances, for instance, when spermatozoa are in weak formaldehyde solution, or in some staining solutions, but the process takes place very slowly and quite irregularly. On the other hand, in the egg-water after five minutes almost all spermatozoa show this *lateral body*.

Fresh preparations of spermatozoa of *Arbacia* in egg-water also show quite clearly the elimination of a substance through the pointed apex of the spermatozoön. This appears as a small granule on the points of almost all spermatozoa. The adhesion of the spermatozoa to one another or to other objects is made by means of this granule.² In the middle of some clusters one can see groups of such granules very refractile, round and close together. For this observation only fresh preparations are to be trusted, because stains may form very fine precipitates which cannot be distinguished from drops of the eliminated substance.

Very probably, under the influence of some chemical substances included in the egg-water (Lillie's fertilizin?) spermatozoa eliminate through the micropyle a sticky substance, which so long as it still adheres to the apex of spermatozoa keeps them agglutinated. After it is lost from the tip, the spermatozoa spread out again in the fluid. I cannot decide if this substance exists as such in the spermatozoön or if it is produced by a secretory process at the moment of stimulation by egg-water, as in the sense of Bowen.³

¹ Here also is the opening observed by Mischer and Ballowitz (*loc. cit.*) and called by the former worker *microporus*.

² Ballowitz, E. (*Zeitschr. f. wiss. Zoöl.*, 1890, Bd. 50, S. 317) called this granule "Spitzenknopf" and noticed its instability (p. 375).

³ According to R. H. Bowen, who adopts the theory of Nassonov concerning the rôle of Golgi apparatus, "Das Golgische Binnennetz und seine Beziehungen zu der Sekretion. Untersuchungen über einige Amphibiendrusen," *Arch. f. mikr. Anat.*, 97 Bd., 136, the acrosome should be a secretory organ and the granule on

The spermatozoa also undergo in the egg-water more extensive alterations, which are expressed by changes in the arrangement of the substances and as the end result we have the formation of the *lateral body* as described above.

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NESTING HABITS OF SOME ANTHIDIINE BEES.

CLARENCE P. CUSTER AND CHARLES H. HICKS.

The habits of the Anthidiine bees have been studied by Fabre (2), Friese (3), Davidson (1), Melander (7), Newberry (8), Hungerford and Williams (6), Hicks (5), and others. Fabre early considered the genus *Anthidium* to consist of two groups, divided primarily on habits. One he termed "cotton-workers"; the other "resin-workers." The former now comprise the genus *Anthidium*; the latter *Dianthidium*. The habits of these bees have not been studied so thoroughly in America as in Europe. Studies of *Anthidium porterae* Ckll. have been made by Hungerford and Williams (6) and Hicks (5), and *Dianthidium sayi* Ckll. by Hicks (5).

The writers are indebted to Dr. T. D. A. Cockerell and to Dr. Francis Ramaley for helpful suggestions and for the determination of specimens; to Dr. Edna Johnson for the determination of some plants; and to Mr. S. A. Rohwer for determining some wasps.

We continued a study of these bees during the summer of 1926. The following is an account of our work.

Anthidium porterae Cockerell syn. *maculifrons* of authors,
not Smith.

Hungerford and Williams (6) state that two females of this bee were observed nesting in a bank of coarse, quartz sand near a colony of bembecid wasps. The down of the cells of the nest was thought to be derived from thistle (*Cirsium*) stems or from the inflorescence. The tunnel of one was 10 inches long.

Most of our study of this species has been done at Point East¹ and at Base Line Lake,² near Boulder, Colorado. The nests have been found both on hillsides and on level ground, usually in bare areas.

¹ A small hill rising above the plains, 3 miles east of Boulder.

² Six miles east of Boulder on the plains.

We attempted to observe whether or not *A. porterae* digs her own nest or uses one already constructed by another insect. Individual females have been observed as long as 15 minutes at a time entering, spending a few seconds within, and then leaving various types of holes. At other times the bee merely looks in and leaves. We have never seen this or any other species of *Anthidium* digging in the ground.

Sharp (9) states that the species of *Anthidium* do not form burrows for themselves, but use suitable cavities formed by other insects in wood, deserted nests of other bees or empty snail shells.

As far as can be ascertained, no one has before observed an *Anthidium* bee to drive away another insect and take the nest, although it has been generally assumed that it uses the deserted nests of another insect, or a convenient cavity.

While observing a wasp, *Odynerus dorsalis* Fabr.,¹ building and provisioning a nest, *A. porterae* was seen to drive away this wasp and appropriate the nest for her own. A few days previous to August 2, a female of this wasp² had been observed working and nesting. During the morning of August 2, I had been watching this wasp excavating and carrying out moist pellets of earth. Shortly after noon she had apparently finished the nest. The following notes were taken in the field near Base Line Lake between August 2 and 5, 1926 (h).³

2 : 50'. See *Odynerus* returning, flying and carrying large lepidopterous larva. She enters hole with it, staying but a minute within, then leaves.

3 : 17' 05''. Back without larva. Flies in wide circles after which alights near hole, tapping edge with antennæ a few times. Enters. Backs out. Flies away. The hole is 5 mm. in diameter.

3 : 38' 10''. Back with another larva which she takes within. Out, and flies away.

3 : 40' 15''. *A. porterae* is seen flying low along ground and entering holes nearby.

¹ Kindly determined by Mr. S. A. Rohwer.

² An account of the habits of this wasp given by C. H. Turner, in BIOL. BULL. 42, 1922, p. 153-172, 6 figs.

³ Initial (h) refers to notes of Hicks; (c) to those of Custer.

3:41' 08". She comes to hole of wasp nest. Enters and remains within.

3:43' 33". Bee still within. Wasp returns, alighting at edge. *A. porterae* is near top of hole with jaws spread in a threatening manner, and head blocking the entrance. Wasp jumps at bee in hole, then flies away. Back and strikes at abdomen of bee as the bee backs out of hole. Bee and wasp leave, fighting. Wasp soon returns, alights near hole, tapping it with her antennæ. Enters and is pulling out one larva as *A. porterae* returns. Bee strikes and tries to bite wasp with her mandibles, a number of times. The wasp flies with larva and drops it 10 inches from hole after which she flies away. Bee then goes inside. Wasp returns but pays no attention to larva which she dropped but comes to hole and looks in for a time. She then flies away.

A. porterae leaves.

3:54' 01". Bee enters hole.

3:56' 52". Goes away.

4:05' 50". Bee returns.

4:06'. Wasp returns. Tries to enter, jumps back and flies away.

4:07' 02". Bee flies away.

4:18' 15". *A. porterae* enters. Believe carried something inside.

4:18' 40". Leaves.

4:20' 55". Bee returns, carrying a load of down.

4:21' 05". Flies away.

4:23' 40". Returns with load of down.

4:24' 15". Leaves.

4:26' 29". Returns with down.

4:27'. Leaves tunnel.

4:27' 01". Wasp returns. Looks in. Goes in and comes out almost at once. Flies a few inches away. Returns. Looks in. Goes in. Out and is ready to go in again when *A. porterae* returns with load of down. She strikes wasp and both fly. The bee follows wasp fighting and striking her while in the air. The wasp circles back to the nest followed by bee, and tries to go into the hole. The bee repeatedly strikes wasp as latter tries to enter. *A. porterae* drives her away, following her out of my view.

4 : 29' 33". Neither have come back.

4 : 31'. Wasp back. Looks in and flies away. Back again soon. Looks in. Goes in. Flies away in a circle. Back to nest. In. Out with some down in mandibles. In again and out with down in mandibles. Six more trips in and out with down. Then out dragging larva by head. Gets larva half out and puts back in at once as though she sees mistake. In and out with five more loads of down. Is now 4 : 35'. Comes out again with load of down at which time bee returns flying at wasp and hitting her a number of times. Bee then enters hole. Wasp is back very soon again. Enters hole in which bee is in but backs out at once. Follows an active four minutes in which the wasp repeatedly flies about hole, returns to it, looks in, flies away a few inches, back, etc. The bee is well up the tunnel blocking the entrance, during this period. The wasp flies away.

4 : 41'. *A. porteræ* is still in the hole but has gone down so that cannot see her.

4 : 42' 55". Bee out. Flies around and back into tunnel. Goes down into cell.

4 : 46' 10". Wasp flying rather high over nest but does not stop.

4 : 48' 20". *A. porteræ* out. Flies a few feet away and back carrying nothing.

4 : 53' 20". Repeats above.

4 : 55' 20". Repeats.

4 : 57'. Bee backs out dragging larva carried in by wasp to the outside. Drops 2 inches from nest. Enters. Out head first. Leaves.

5 : 01' 02". Back with down.

From this time until 6 : 30 the bee carried 17 loads of down. The wasp was not seen again. The next day the bee carried 18 loads of down. The day was partly cloudy. On August 4, by 10 o'clock the bee was collecting pollen and by 9 : 15 A.M., August 5, the nest was complete with the tunnel filled with pebbles to the surface of the earth (h).

Anthidium bees have been observed by many students to use down from various plants growing in the vicinity of the nests. Fabre (2) found that some would even readily make use of that

from introduced plants. He states that *A. diadema* will take the cotton for its work from any suitable plant growing near its nest. This bee nests in hollow reeds placing as many as ten cells in one stem. The most we have found here have been two in one nest, more usually one. This is due to the fact that most of ours have probably taken over wasp nests providing space for but one or two cells.

According to Friese (3) certain European *Anthidia* obtain their down from the leaves of different hairy plants, among which are especially such plants as *Stachys*, *Ballota*, *Cydonia*, *Verbascum*, *Populus* and *Gnaphalium*.

A. porteræ gathers down from the underside of the leaf of *Cryptanthe gracilis* Osterh.,¹ by scraping it up towards the apex with her mandibles.

After visiting several leaves she then holds the ball gathered with the mandibles between the thorax and front legs and flies with it thus to the nest. A given individual usually returns to the same group of plants to obtain the down. One bee averages two and one-half minutes in securing a load (c).

We have observed a bee to go a distance of over 50 yards in securing the down although some others have been seen to go but 5 yards away while one at White Rocks went but 3 feet from the nest.

In August, 1926, *A. porteræ* was observed to gather down from the stems of live *Artemisia canadensis* Michx.¹ One female was observed while she went from plant to plant visiting a large number on one collecting trip. This bee scraped the down in much the same manner as those observed working on *Cryptanthe*. It would seem that this latter plant is much better for the bee as it has more down and requires less work to obtain it (h).

On July 20 and 21, 1926, notes were taken on the provisioning of a cell, an extraction of which follows (h).

One nest was watched continuously for a number of hours. The opening to the tunnel was slightly enlarged so that the bee could be seen working within. She would return to the nest carrying the down with her mandibles, enter, and arrange it

¹ Kindly identified by Dr. Edna Johnson.

into a cell shaped for the reception of the pollen. She usually turned herself around once and sometimes two or even three times in the placing and working of each load.

In returning to the nest the bee came direct, leaving in the same manner. Although the weather was partially cloudy the bee carried 26 loads of down in two days, an average of six minutes and thirty-eight seconds for each load.

The time of individual trips varied from 4' 49" to 16' 05". The time spent within the nest, arranging the down varied from 1' 10" to 2' 20". The time taken to get the down is determined by weather conditions, distance secured from nest, time of day, etc.

At the time of provisioning, the cell had an inner cavity, a little larger than the bee. She began at 1:40 to carry pollen and between this time and 3:30 carried 6 loads. The time spent within the nest averaged about 2 minutes. On arriving, the bee opened the top of the cell, entered head first, came out, and backed in, forcing the abdomen inside. While here, she removed pollen from the ventral scopa and deposited it in the bottom of the cell. Before leaving the nest, she always partially closed the cell by raking the down together at the orifice. This may make it more difficult for parasites to find or enter the nest.

After depositing the pollen, the bee was not seen to enter the cell again until she had returned from another collecting trip. If nectar was deposited after each collecting trip, it of necessity was deposited first when the bee had her head within. However, the first pollen was seen to be dry and it was not learned if separate trips were made to gather nectar or not. The final food for the young is very moist (h).

After provisioning, laying the egg and closing the cell the bee fills up the tunnel, usually with pebbles. These may be selected from a small area over 9 yards from the nest, in rare instances, but more usually from 1 to 3 yards away. A number of times the bee has availed herself of the pebbles of a large red ant nest (*Pogonomyrmex occidentalis* Cresson).¹ The number of pebbles used is at least enough to fill the tunnel above the cell even with the surface of the ground. The size of the tunnel and the

¹ Kindly determined by Professor T. D. A. Cockerell.

distance above the cell varies. From 135 to 300 pebbles are used, 250 being about the average.

Sometimes a bee loses its hold or attempts to carry a pebble too heavy and is forced to drop it. She then goes at once to the place, from which she secured the one dropped, for another. However in one instance, upon dropping the pebble midway to the nest, the bee alighted at the spot, seized another, and continued on her way. The bee usually drops the pebble from above the hole into the tunnel but on a few occasions she was observed to alight on the edge before dropping it in. Sometimes the pebble fails to hit the hole and remains close to the edge. While filling the hole the bee frequently enters to arrange the pebbles. In doing this she turns them with her mandibles and fits them into position snugly. In only one instance, after the bee had gone inside to arrange the pebbles, have we observed her on leaving to rake in some pebbles and soil from near the edge with her mandibles and fore legs.

On July 24, 1926, found bee filling tunnel. She was watched until the end of this period. The following is an extract from more detailed notes taken in the field (h).

At 8:50 A.M. found *A. porterae* entering hole in ground with piece of dry dirt. The hole was large enough to see inside and it was noted that only a few pieces had been deposited for the down was not covered. When first observed the bee was carrying material from a place 12 feet east of nest. She carried 10 loads in an average time of 12 seconds. Each time the bee alighted on the ground and selected the piece of dirt. These chunks varied somewhat although the average was quite uniform.

After she carried a few more, a circle of stones was placed about the nest while she was after a load. The stones were about the size of a hen's egg and were 3 inches from hole. On returning she circled 3 times about the hole, then flew 10 feet away, returned, alighted on ground, returned and entered hole. She then went to collect again and continued, returning direct and without further hesitation, paying no more attention to the change. After 15 more loads she changed her place of selecting materials to 18 feet south of nest. The bee, after every few loads, went and arranged them. After 56th trip changed back to position

12 feet east. After 113th trip (9:47) bee went to feed on *Psoralea* plant. She was followed and was found to visit 83 flowers in 6 minutes. She then carried 3 loads into nest and fed again, visiting 15 more. The 139th trip ended at 10 o'clock.

The bee had no sooner alighted, after the 155th trip than a male met her. The period of mating lasted 25 seconds after which the female went on with her work as before. In one instance she carried in a piece of old stem which was so long that it was bent back to get into the hole.

She made in all 240 trips between 8:50 and 10:26 at which time the tunnel was filled even with the surface. This bee carried in almost entirely pieces of dried mud; all others observed have used mainly pebbles. The nest was dug up and consisted of two cells. This nest was of the nature of those made by *Odynerus dorsalis* Fabr. The lower cell was opened and contained a small larva of *A. porteræ* which later died. The upper was not opened until in September at which time a cocoon had been formed containing a live, mature larva. In this instance one mating took place after the egg of the two cells had been laid, both eggs hatching. It is probable that *A. porteræ* mates more than once. Other matings have been observed to take place while the female was collecting pollen for the cell. Once a female of this species was found to be mating with a male, *A. porteræ amabile* Ckll., a variety with more red on the abdomen (h).

The mass of pollen, semi-fluid in consistency, of the cell of *A. porteræ* is 10 mm. long, flat on the top surface and curved on the base. The egg lies flat on top of the mass of provision. A larva $2\frac{1}{2}$ mm. in length was found to be partially embedded in the food. The exact larval period has not been ascertained although a number of cocoons have been formed in the laboratory. The larvae of those opened never developed much further and it was thought that this might be due to the pollen drying out as well as to possible mechanical injury (h).

The size of the entire cell including the down varies a little, due to the size of hole in which the down is placed. One which was about average in size measured 17 mm. in length, being 12 mm. wide at the bottom and 10 mm. wide at the top. Pellets

of excrement mixed with down were all about the down and between the cocoon and the outside (h).

The outer surface of the cocoon has a thin layer of rather light, loosely woven silk. Removing this the denser, darker cocoon is found. An average cocoon measured 10 mm. in length and 5 mm. wide, being oval in shape with the ends somewhat flattened. The anterior end bears a mammillary point which externally contains an opening. This point is not so long nor so conspicuous as the one on the cocoon of *Dianthidium sayi*. One large cocoon measured 12 mm. in length and 7 mm. in width. The cocoons found have been reddish brown. One of the cocoons described, held to a bright light showed the outline of the larva within (h).

We made certain investigations in which we attempted to find if *A. porterae* would carry pebbles indefinitely to the nest. While the bee was away for a few seconds to obtain another pebble we removed the one, with a pair of forceps, she had last dropped in. Thus the nest was kept continuously empty or partially so.

On July 27, 1926, at Point East at 3:15 P.M. we found a bee carrying large pebbles from the nest of the red ant (*Pogonomyrmex occidentalis* Cress.) 9 yards away. The nest at that time was about half full. We removed 175 pebbles by 5:20 P.M. when the bee quit working for the day. The nest was watched until 7:00 P.M. and from 7:15 A.M. the following morning but the bee was not there at either time and no pebbles had been carried in the interval. She started again to carry pebbles at 7:55 (July 28). From 7:55 to 8:20 she carried 100 stones, which is an average of 4 stones a minute. Later in the day (10:00 A.M.) she was found to be averaging 8 a minute, probably due to the increased intensity and heat of the sunlight. She carried 311 pebbles before noon, having fed early in the morning, suddenly stopped and was seen no more. Although the nest was watched for days, no more pebbles were carried. The total number carried was 741. The bee was not frightened away and apparently quit voluntarily, leaving a number of pebbles in the nest.

To another nest a bee carried at least 400 pebbles and suddenly left, although the hole was kept partly empty as in the other case. Before stopping, in each instance, there was no apparent slowing

down of the work, nor cloudy or cool weather. The bee stopped during weather conditions very favorable for work.

In two instances, I have found the nests of some species of *Anthidium* in which the tunnel was unusually short, and over and above which she had carried a mound of pebbles instead of just filling even with the surface, as in the case of nests having longer tunnels. In one instance 135 pebbles and in the other 75 had been carried, more than enough to fill the tunnel (h).

From our investigations it would seem that *A. porterae* will not carry pebbles indefinitely even under ideal weather conditions.

Whenever the cell becomes damp and mold develops, as sometimes after rain, the nest is deserted.

Both males and females have been found resting in the dry pods of yucca stems during rainy or cloudy weather, in cavities in decayed wood or resting, perched bird-fashion on stems during the night.

During the day when the weather is fine the males dart from flower to flower and are often seen hovering humming-bird fashion in the air. Part of the day is spent in feeding and part in searching for the female. He often darts upon the female while she is working, copulation taking place on the ground. The duration of this period is about 30 seconds. As yet we have reared no parasites.

SUMMARY.

1. No females of *Anthidium porterae* have ever been found digging a nest.

2. On one occasion she drove away a nesting wasp, *Odynerus dorsalis*, and used the nest for her own.

3. The nests found in which the cells of the bee are placed have in general been such as are made by wasps or other insects nesting at the time.

4. *Anthidium porterae* is solitary and is not found nesting in close association as *Dianthidium sayi* and others.

5. The cell is constructed of down and the tunnel above filled to the surface of the ground, usually with pebbles.

6. When the stones are removed artificially from the tunnel, *Anthidium porterae* probably will not replace them indefinitely even under ideal weather conditions.

Dianthidium sayi Cockerell syn. *interruptum* Say. (nom. preocc.).

The bees of the genus *Dianthidium* have been known to construct nests of resin on rocks, in stems of plants, in deserted snail shells and other places, but, so far as we know, seldom in the ground as does *Dianthidium sayi*. We have found no reference to the nesting habits of this species except that in 1926 by Hicks (5). This latter information was derived from a number of cells dug up from a small area at White Rocks, during fall of 1925 near Boulder, Colorado. We have obtained our field notes during the summer of 1926 mainly at this locality where two colonies, one hundred yards apart, were found and studied.



The six colonies of *D. sayi*, observed during the latter part of the summers of 1925 and 1926, were all located on hillsides that face south and slightly east. Scattered over these hillsides were tufts of *Bromus briziformis* Fisch. and Mey.,¹ to the roots of which some of the resin cells were attached. We find that the species may nest as a colony of from eight to fifty or more females, although one apparently solitary female was found nesting in a vertical clay bank. The colony is at its maximum activity during the month of September.

The picture shows a small colony of *D. sayi* on a grass-covered mound of sandy soil one yard in diameter. Base rock surrounds this on all sides, the nearest soil being five feet away. Tall

¹ Kindly determined by Dr. Francis Ramaley.

stems of *Bouteloua oligostachya* (Nutt.) Torr. and *Bromus briziformis* Fisch. and Mey., rise a foot or so above the colony; thus chaff, small sticks, etc., are abundant. The nests were marked, as seen in the picture, 1 to 16 by small numbered strips of paper securely weighted with sand. Nest 7 has been plugged up. The picture shows bee *II*, whose thorax is white for identification, very near and to the left of this nest. She is standing on her hind legs with the fore legs on the bank just above the nest. Nest 4, hidden from view, is a foot behind 2 while nest 14 is a quarter inch above 5. Nests 11, 12, 13, 15 and 16 are hidden from view in the grass to the left of 14. Nests 7 and 8 are about twenty-five inches apart (c).

All bees concerning which notes were taken were marked with waterproof white paint. Thus the thorax of the bee seen beside label 7 is white. Bee *C*, on whose habits many important notes were taken, was marked as follows: (1) Left half of dorsal surface of last abdominal segment white; (2) A black area present on left side of fourth dorsal abdominal plate completely surrounded by a yellow tegumentary band which area, on right side of same abdominal segment, was not completely surrounded; (3) Right postero-lateral quarter of mesothorax dusty, probably due to some resin sticking there at one time and dust adhering to this (c).

One bee was seen to have two nests in the process of construction at which she worked more or less alternately. Later, five such bees were observed. These were shown to and verified by Hicks. The following short extract illustrates this habit in the case of one bee: Bee *C* is constructing two nests which are nine inches apart. Into one nest she carries pebbles, lumps of dirt, chaff and such sticks as she can handle with her mandibles. Then she flies directly to the other nest and does the same thing there. Thus she carries the following number of these articles

To Nest 1:	Then to Nest 2:
6.....	6
4.....	5
9.....	0
(Then a male arrives and they mate while she has a stick in her mandibles (60 seconds). He leaves and she continues her work after carrying this stick to nest 1.)	
23.....	0
(She flies to flowers of <i>Grindelia</i> several yards away, and feeds there for ten minutes; then she returns to work as before.)	
5.....	1
5.....	0
Here she flies to the fields. One of the sticks is one and one half inches long (c).	

The following account shows this habit in another individual: Bee *D* goes into nest 3 for fifty-five seconds. After going out and in several times, she leaves, flies slowly over to nest 4, nine inches away, and goes in at once. Presently she leaves this nest and flies to some nearby flowers to feed. In one minute she arrives at nest 3, leaves after a few seconds, and flies slowly over to nest 4 which she enters. After one half minute, she leaves, feeds for five minutes and goes into nest 3. She comes out, three quarters of a minute later, goes into 4 and spends one half minute there before leaving for the fields to feed (c).

It has not been observed whether or not *D. sayi* digs the hole for its nest and so it can not be proved that one bee has attended to two holes from the first. However, the proximity of the nests to one another indicates that it does the former. It might easily be that each summer, when the colony began to nest, there was but one bee to each hole. Then when some of the builders died, before completing their nests, others of the colony would appropriate these deserted nests and finish them along with their own. The following examples are taken from the notes to give evidence that temporarily deserted nests of *D. sayi* are readily taken over by other members of the colony:

Example 1.

Sept. 14, 1926. While bee *I*, the owner of nest 8, is in the field, another member of the colony comes and goes into the nest. Thirty-five seconds later, bee *I* arrives and goes into

nest 8 also. Presently the owner backs out and is followed closely by the intruder. The former alights on the intruder's thorax, biting with the mandibles at the junction of head and thorax. A short, lively tussle follows, after which the intruder escapes and the owner goes on working.

Example 2.

Sept. 17, 1926. Bee *D*, which has been constructing nests 3 and 4, has now spent ten minutes carrying chaff, etc., into 4. In the meantime, bee *I*, which has been working on nest 8, arrives from one of her numerous trips to the field and goes into nest 3. She at first makes regular trips carrying soil out of this nest. Then she seems to be carrying pollen in. The owner of nest 8 has been working on 3 all day and has but rarely visited 8. The owner of nests 3 and 4 has continued to carry chaff, etc., solely into 4, eventually plugging it up. The next day the owner of nest 3 returns. The owner of nest 8 sees her return and so is now working as before on 8 (c).

Five bees by this time (Sept. 17) have been seen repeatedly to visit two nests apiece. To summarize, they are: (1) Bee *C* to nests 1 and 2; (2) Bee *D* to nests 3 and 4; (3) Bee *E* to nests 5 and 14. (4) Bee *I* to nests 8 and 3 (to 3 only to-day); (5) Bee *H* to nests 6 and 7. There are eight bees working in the colony. Of the sixteen nests, nine are open and seven are plugged, but of the latter, numbers 7, 15 and 16 are not being worked upon by the bees. Thus the eight bees of the colony are working on thirteen nests, and three of these bees are seen to work on but one nest apiece (c).

On September 26, we dug up the colony and found 151 completed cells from the 16 nests, which is an average of 9.44 cells to a nest. This was practically the end of the nesting season, for at this time and later the colonies of *D. sayi* were deserted.

It is interesting to note, from a standpoint of comparative entomology, that a wasp, *Sphex varipes* (Cress.)¹ has been observed to be taking care of two nests also (h).

From these facts and others in our study of bees, we have found that there is sometimes considerable variation in the habits of different individuals of a given species.

¹ Kindly determined by Mr. S. A. Rohwer.

The picture shows bee *H* in one of her numerous visits to the plugged hole 7. Her action suggests that a bee retains the memory of her nest for at least a week after it has been completely plugged up and that her visits are made to it to insure its security from enemies. A brief extraction from my field notes illustrates her action: "Bee *H* goes into nest 6 for one and one half minutes, comes out and goes over to plugged hole 7. She spends about ten seconds here. Upon arriving at 7, she first puts her head down against the plug, rubs it with face and mandibles and scratches weakly with fore legs for three or four seconds. She then stands erect before the nest on her hind legs with her fore legs against the bank above the plugged hole (see picture). After standing thus motionless for about five seconds, she flies away to the fields. She repeats this procedure many times daily in like manner. Only occasionally does she first go to nest 7 and then to 6" (c).

While bringing out soil from within the nest, after the tunnel has been constructed, bee *D* has been observed to back out, raking it with her forelegs. She usually leaves this within an inch of the entrance although in a few instances she took it out in this manner as far as six inches, and once ten inches (c).

D. sayi obtains the resin used in the construction of the nest from the small sunflower, *Helianthus petiolaris*. She obtains this resin, with her mandibles, from the stem and partially or totally dried leaves which have small droplets and plates of it distributed over them (c).

Fabre (2) studied "resin workers" for many years but states that he never saw a bee get resin. Friese (3) states, in reference to the source of resin used by a species found in Europe, that it is probably obtained from the buds of the pine. Hacker (4) inferred that *Megachile rhodura* used resin for its nest since he observed members of both sexes visiting a *Eucalyptus* tree, from which resin had oozed, and rasping the resin with their mandibles. However, he did not find *M. rhodura* nesting. Melander (7) presumes that the resin used by *Anthidium texanum* Cresson was obtained from cedar. Hungerford and Williams (6) say, in their account of *Dianthidium concinnum* (?) Cresson: "The nest of this insect is composed of pebbles glued together

with resinous cement which may be derived from the stems of *Helianthus* which are often infested with a small lepidopterous borer that causes an exudation which usually attracts a host of Hymenoptera."

The following field notes were taken, giving the resin activities of bee *C* during one hour:

12:57 $\frac{1}{2}$. Arrives with resin in mandibles at nest 2. Kneads resin with mandibles.

1:00. Obtains a piece of chaff an inch away from nest and inserts it into the resin which by now she has shaped into a plug over the ends of the pieces of chaff projecting from the burrow.

1:06 $\frac{3}{4}$. Carries piece of chaff to nest 2.

1:07. Obtains small stick and flies with it to nest 1.

1:07 $\frac{1}{2}$. Flies away. I follow her. She goes to some flowers of *Grindelia* and feeds.

1:09 $\frac{1}{2}$. Flies to some dried plants of *Helianthus petiolaris*. Picks a plant without many red and black ants present and scrapes resin off with mandibles. She visits three plants.

1:12 $\frac{3}{4}$. Arrives from fields at nest 2. Has in her mandibles a load of resin about one fourth as large as head. Distributes resin over ends of chaff projecting from the entrance.

1:21. Flies to nest 1.

1:21 $\frac{1}{2}$. Leaves nest 1. Goes directly to sunflower plants and secures resin.

1:24. Arrives at nest 2.

1:25 $\frac{1}{2}$. Leaves nest 2.

1:30. Arrives and leaves after two seconds. I follow her to several flowers of *Grindelia* where she feeds.

1:32. Arrives at nest 2 and leaves, after two seconds, for the fields.

1:34 $\frac{1}{2}$. Arrives at nest 1 with white resin in mandibles. Works here with the resin in the construction of a plug.

1:36 $\frac{3}{4}$. Obtains large pebble two inches away and inserts it into resin of nest 1.

1:38. Flies to nest 2 and works with plug there.

1:42. Carries stick to plug at nest 2.

1:42 $\frac{1}{2}$. Leaves nest 2 and feeds at flowers of *Grindelia* and of *Helianthus petiolaris*.

1:46. Arrives at nest 2. Leaves after two seconds and again feeds.

1:48. Arrives at nest 2. Walks all over colony looking into several nests and returns to this one.

1:49. Flies away.

1:51. Arrives with resin at nest 2.

1:51 $\frac{1}{2}$. Obtains piece of chaff and takes to nest 1. Gets stick midway between nests 1 and 2 and carries it to nest 2.

2:01. Leaves nest 2 and obtains resin after feeding on flowers of *Grindelia*, etc. (c).

The entrance to the tunnel, after the completion of the cells, is closed by a plug of resin, chaff and other materials. From several plugs taken out, we find that the resinous part is about 2 mm. thick. Usually, in constructing the plug, the bee first places a few pieces of chaff, sticks, etc., in the entrance and then builds a resin cap over the projecting ends. Beyond the plug, towards the cells, the tunnel is often empty and may be coated with resin.

One of us (Hicks) (5) has described the cells of *D. sayi*. Last summer it was not known whether all the materials found in the resin had been carried there by the bee or were there by chance. Observations this summer show that she obtains the chaff, sticks, etc., which are found mixed with this resin, from the vicinity of the nest. In some instances she has been found to carry fifty or more pieces of chaff, etc., into one tunnel until it was filled. After this she finished the nest with a resin plug. The resin cells are often found attached to the grass roots in similar fashion to peanuts on the stem.

D. sayi varies considerably in the distance which she carries the stones, dried mud, etc., with which she constructs the nest. Bee *J* (attending to nest 9) is seen to make many trips with plates of dried mud from an old mud puddle, six yards away, to her nest. Bee *E* (attending to nests 5 and 14) is seen to carry many large pebbles to her nest from a sand pile eleven yards away. She places these in nest 14 after having plugged up 5 (c).

D. sayi may also obtain chaff and sticks from a distance varying from three inches to three yards away. One individual selected material near the nest but did not repeatedly obtain it from the

same place; another repeatedly visited a more distant source. This latter was usually found to reject several pieces of chaff, etc., before taking one to the nest (h).

The female of this species (bee C) was seen to mate twice within three days. The first mating, on September 13, is recorded in field-notes given on page 270. The second mating is given in the following extraction: On September 16, bee C leaves nest 1, which is now almost totally plugged up with chaff, etc., and flies to some flowers of *Grindelia* to feed. She then flies to some small sunflowers nearby and feeds there also. Finally she goes to a flower of *Lygodesmia juncea*. While feeding there, a male arrives and they mate. This mating takes sixty-five seconds. The female is grasping the stamens of the flower with the fore and mid legs and is touching the hind legs repeatedly on those of the male. This is apparently the first record of a wild bee mating more than once (c).

The male has been observed to await at the colony the return of a female. As soon as she arrives, he darts at her to effect copulation and in some cases they collide in mid-air, both falling to the ground. The female, with partially outstretched wings, rests motionless except for a slight movement of the hind legs on those of the male. The male has the female grasped around the third abdominal segment with the forelegs and beneath the abdomen with mid and hind legs while the tips of his antennæ are bent forwards and outwards. His only movement is a relaxation and contraction of the abdominal segments and a slight waving of the tips of the antennæ. The average time for copulation, from seven examples, is very close to a minute (59.7 sec.). We have observed that mating may occur both at the colony and at the flower while the female is feeding. Although the male is absent from the colony most of the time, nevertheless the majority of the matings observed have taken place at the nest.

Mating probably does not interfere with the nest-building activities as evidenced by the field notes on the first mating of bee C. Furthermore, a female has been seen, returning laden with pollen, to mate, after which she immediately went into the cell, deposited the pollen and went on with her work.

D. sayi has been observed to feed on the flowers of the following plants:

- (1) *Grindelia squarrosa*.
- (2) *Helianthus petiolaris*.¹
- (3) *Sideranthus spinulosus*.¹
- (4) *Lygodesmia juncea*.¹

After a rain, which partially filled the entrances of some holes, we completed the closing of these with wet sand to observe the later action of the owners. One, entrapped within the nest, used the mandibles to remove the plug; another, returning from the fields, also used her mandibles to gain entrance. The latter was observed to place head and mandibles into a small excavation, which she had formed, and to walk in a clockwise direction around the hole with the tip of the abdomen describing a circle. The forelegs were practically useless in this process. The abdomen was not used in this case to pack the soil in repairing the walls, as has been observed in a bee of the genus *Augochlora* under similar circumstances.

D. sayi has not been found feeding beyond a radius of one hundred yards from the nest. However, of six marked females released four hundred and fifty-five yards from the colony, four returned within five hours. The shortest time required to return this distance was twenty-one minutes. Two specimens released one hundred yards from the nest returned shortly, the first within fifteen minutes, after feeding along the way (c).

The only parasite of *D. sayi* which has been bred is a fly of the genus *Villa* (Anthrax),¹ Hicks (5). Some mutillids and chrysids may be parasitic since they have been frequently observed to enter the open or partially plugged-up nests.

SUMMARY.

1. Several instances of a female bee, *Dianthidium sayi*, working alternately on two nests in the process of construction, have been found. This has also been observed in a species of wasp, *Sphex varipes*. Apparently these are the first records of this habit.

¹ Determined by Professor T. D. A. Cockerell.

2. Resin, used in the construction of the nest, is obtained from the leaves and stem of the small sunflower, *Helianthus petiolaris*.
3. *D. sayi* is one of those wild bees whose members nest in close association with one another.
4. A female of this species was seen to mate more than once.
5. *D. sayi* seems to be nearly free from parasitism.

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ORIGIN OF THE MESODERM AND BEHAVIOR OF
THE NUCLEOLUS IN REGENERATION
IN *LUMBRICULUS*.¹

LEONARD P. SAYLES.

INTRODUCTION.

The microdrilous annelids have been the center of considerable interest on the part of students of regeneration particularly from the point of view of the origin of the cells which form the new tissue. While the production of new endodermal and ectodermal structures from cells of the corresponding old parts is generally accepted, the origin of the mesoderm in the bud has been a point of contention. Hepke ('97) in *Naids*, von Wagner ('00 and '06) in *Lumbriculus* and Abel ('02) in *Tubifex* and *Nais*, among others, are of the opinion that the new mesodermal structures both in anterior and in posterior regeneration are of ectodermal origin. Randolph ('92) in posterior regeneration in *Lumbriculus* reports their formation from comparatively unmodified cells of the old mesoderm to which she gives the name "neoblasts." Iwanow ('03) in *Lumbriculus* and Krecker ('10) in *Tubifex* and *Limnodrilus* verify her conclusions. They find that in anterior regeneration, however, cells from old specialized mesodermal structures form the new portion. One of the features in the descriptions of the cells of the bud region by these workers is the prevalence of large nucleoli in the cells involved in the regenerative activity. Krecker ('23) gives a rather complete description of the origin and migration of the neoblasts in posterior regeneration. Studies on the origin of the new mesoderm in regeneration together with observations on nucleolar changes in these and other tissues are reported in this paper. For invaluable advice on this work I am indebted to Dr. J. W. Wilson of Brown University at whose suggestion the problem was first undertaken.

¹ This paper forms a part of a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Brown University, June, 1926.

MATERIALS AND METHODS.

The species used in this work was identified for me by Professor Frank Smith, of the University of Illinois, as of the genus *Lumbriculus*, and probably the species *inconstans* Smith. Anæsthetization was avoided as far as possible. When it was necessary, a one eighth of one per cent. solution of chlorethane or a four per cent. solution of alcohol, redistilled in glass, gave equally satisfactory results. For histological fixation Zenker's fluid or Bouin's Picroformol were used; best results were obtained with the latter. It was not necessary to anæsthetize the worms first. An individual to be fixed was placed on a glass plate and straightened against a glass slide. Another slide was then pushed up until it came in contact with the worm, care being taken not to crush it between the slides. In this process only a very small amount of water was used. The fixing fluid was then slowly added and its surface tension, as it was drawn underneath the two slides, held the worm firmly in place during the preliminary fixation. This method is a modification of one described by Welch ('13). As a stain Heidenhain's iron alum-hematoxylin method was used for the most part, though preparations were also made with Delafield's hematoxylin and eosin, used both as a direct and as a regressive stain. On carefully controlled regressive staining the chromatin granules were stained with the hematoxylin while the nucleoli took the eosin, indicating that they were true nucleoli or plasmosomes.

NUCLEI AND NUCLEOLI IN THE CELLS OF UNINJURED INDIVIDUALS.

As this paper is to deal at some length with the cytological changes, particularly nuclear and nucleolar, which occur during regeneration, it is perhaps advisable first of all to discuss more or less completely the conditions which exist in uninjured individuals. Under this heading the usual appearance of cells in the hypodermis, setigerous glands and intestine, including those differences which may exist at the growing region of the tail, will be described.

Hypodermis.

In all the epithelial cells of the hypodermis the nuclei are quite large, all gradations from an oval type (the average measurement of twenty-five of which is $3.3 \times 5.8 \mu$), to the practically spherical one (averaging about 4.2μ) are to be found. In each a very minute nucleolus, appearing as a mere dot under a magnification of $1,000 \times$, is visible (Fig. 1). Cells of this type form all of the hypodermis from the second segment to the growing region of the tail. At this end, just anterior to the place where the basement membrane is discontinuous due to the formation of the ventral nerve cord, the hypodermal cells are slightly elongated. The nucleoli are slightly larger than in the cells located more anteriorly (Fig. 2). Cells of this type are probably intermediate stages between the active ones of the growing region and the typical hypodermal cells found in the older part of the body. Such an elongation of the cells becomes even more marked where the nerve cord is forming with the result that the hypodermis in the growing tip is two or three times as thick as in the rest of the body wall. This elongation of the cells is most marked ventrally just posterior to the end of the nerve cord. The thickening of the hypodermis includes the dorsal and lateral portions as well as the ventral but it does not extend as far anteriorly on the dorsal side as elsewhere.

Bülow ('83), in his discussion of the normal growing region, describes this thickening, but makes no mention of any difference in the nucleoli of this region. In posterior regeneration, however, Krecker ('10) describes a marked enlargement of the nuclei and nucleoli of the ventral ectoderm. In the normal growing worm the nuclei are larger near the growing nerve cord and a short distance posterior to it than they are elsewhere (Fig. 2); the average size of these nuclei is $3.9 \times 7.4 \mu$. The cells in which such a difference is apparent are the ones involved in the formation of the new nerve cord. The nucleoli, on the other hand, are enlarged throughout the entire tip and to a slight degree for a short distance from it. This difference is most decided in the ventral cells, in which the nucleoli may be nearly 2μ in diameter, but average between 1 and $1\frac{1}{2} \mu$.

Setigerous Glands.

Each pair of setæ is embedded in a mass of cells derived from the ectoderm and to them are attached secondarily mesodermal cells which form their musculature (Penners, '23). The setæ themselves are formed by the ectoderm cells, according to the description of Bergh ('90), more recently corroborated by Penners. In the earlier stages of the formation of the setigerous glands in the growing region of the tail most of the cells possess oval nuclei of about the same size as those of the neighboring ectoderm cells and nucleoli measuring between 1 and $1\frac{1}{2}\mu$. As muscle fibers are added to the outside of the gland cells it becomes more difficult to study the latter but it seems certain that most of them have large nucleoli for a number of segments from the growing region. In the older part of the worm, however, the nucleoli are smaller, for the most part mere dots (Fig. 3). Occasionally a few cells are to be found possessing fairly large nucleoli (1μ); these cells may have been active in the formation of a new seta to replace one which had been lost.

Alimentary Canal.

The cells of the alimentary canal in the first segment are typical hypodermal cells, of cuboidal shape with round nuclei 4 to 5μ in diameter and nucleoli which appear as mere dots (Fig. 1). These cells, unlike those of the rest of the digestive tract, are without cilia but covered with a cuticle, as are those of the hypodermis (Pointer, '11). In the second somite the cells are more elongated and the nuclei are of the oval shape ($3.6 \times 6.9\mu$) typical of most gut cells of this species. This segment is a transition zone between the first and third as far as the nucleoli are concerned. In the latter the nucleoli are fully $\frac{1}{2}\mu$ in diameter (Fig. 4).

These same measurements hold for both the nuclei and the nucleoli in the cells of the next few segments. At about the ninth or tenth segment, however, cells with larger nucleoli appear. These cells increase in abundance until in the vicinity of the thirteenth practically every nucleolus measures at least 1μ (Fig. 5). There is no corresponding enlargement of nuclei. A feature which first appears in this region is the occurrence of

two nucleoli within a single nucleus. This is a phenomenon which is rather infrequent in this species except in regenerating individuals. For brevity, they will be spoken of as "double nucleoli" but this meaning of the words should not be confused with that of earlier workers, particularly Montgomery ('98), whose "double nucleoli" were individual nucleoli made up of two types of material. While no unquestionable cases of double nucleoli have been observed in the first eleven segments, posterior to this region, where the nucleoli are larger, cases of this sort are occasionally found. Counts made on several uninjured individuals indicate that the average frequency is about 3 per segment.

This larger type of nucleolus is typical of the gut cells for a considerable distance. Then there is a gradual decrease in size. In some individuals it seems to be at considerable distance from the anal segment—even 30 segments or more—while in others the larger nucleoli apparently persist at least 15 or 20 segments farther. In this posterior portion of the gut they are hardly more than mere dots in the nuclei (Fig. 2).

Mesoblasts.

In the ventral portion of the coelom in the growing tail region, cells are present which are presumably derived from the mesoblasts of the developing individual (Wilson, '89 and '92). These cells, which produce the mesodermal structures in the newly forming segments, possess large nuclei (about $4 \times 8 \mu$) and nucleoli (about 2μ) (Fig. 2).

Large nucleoli are present, therefore, in the cells of the middle portion of the gut, in those of the hypodermis of the growing tail region and in the mesoblasts. The largest of these are in the cells of the ventral region of the ectoderm, which are forming the new nerve cord, and in the mesoblasts, from which the new mesodermal structures are derived. The significance of these facts will be discussed under a later heading.

ORIGIN OF NEW TISSUE IN POSTERIOR REGENERATION.

Endoderm.

In posterior regeneration in microdrilous annelids the majority of investigators, especially more recently, are agreed on the method of origin of the endodermal structures. Krecker ('10) in *Tubifex* and *Limnodrilus* removes a portion of the intestine after cutting a worm and finds that by a proliferation of cells in the old intestine growth takes place until it comes in contact with the ectoderm. He says (p. 411) that "mitosis and amitosis both occur," using as a criterion for the latter "the frequent occurrence of double and elongating nucleoli." He finds that cell division may take place one or two segments anterior to the wound, but when it "was seen at such a distance it was always found to be mitotic" His observations are verifications of statements to the same effect made by Iwanow ('03). A study of individuals which have been regenerating for various periods reveals that mitoses may and commonly do occur even eleven or twelve segments from the wound region. The distribution of mitotic figures and double nucleoli in worms regenerating for different periods is given in Tables I. to VI., inclusive.

TABLE I.

1 DAY OF REGENERATION.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli	8	8	7	7	10	6	4	4	5	3	2	2	2	3
Mitoses	0	0	0	0	1	0	0	0	0	0	0	0	0	0

TABLE II.

2 DAYS OF REGENERATION.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli	25	31	20	25	21	25	23	20	22	23	19	18	6	2
Mitoses	10	8	4	3	7	4	3	3	2	5	2	2	0	0

TABLE III.

3 DAYS OF REGENERATION.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli	26	21	34	25	27	23	25	21	23	17	9	9	7	3
Mitoses	14	2	5	2	5	3	3	0	2	5	3	1	1	0

TABLE IV.
4 DAYS OF REGENERATION.

Segment from the wound.....	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli.....	26	26	53	46	32	19	15	8	8	7	6	3	1	2
Mitoses.....	5	6	4	7	6	1	0	0	1	1	1	1	0	0

TABLE V.
5 DAYS OF REGENERATION.

Segment from the wound.....	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli.....	44	37	41	33	24	18	18	9	9	6	5	6	3	2
Mitoses.....	6	5	7	3	4	1	4	3	1	1	0	0	0	0

TABLE VI.
6 DAYS OF REGENERATION.

Segment from the wound.....	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli.....	20	23	18	17	20	15	16	7	5	3	4	2	1	3
Mitoses.....	3	2	4	1	1	0	1	0	0	0	1	0	0	0

In making these counts it is necessary to adopt some criterion for double nucleoli. These figures, therefore, are for cases where a nucleus contains two nucleoli in approximately the same focal plane of the microscope. In order that the personal equation involved in making the counts may be made as negligible as possible, instances of dumbbell-shaped or elongated nucleoli are not included. Records are not given for segments beyond the fourteenth from the wound since but 2 or 3 double nucleoli per segment, the number ordinarily found in an uninjured worm, are to be found in these. For mitotic figures all stages from the appearance of the chromosomes in the prophase to the late telophase are included.

As already mentioned, the gut cells of these worms ordinarily have nucleoli of considerable size, averaging about 1μ in diameter, and the oval nuclei measure on the average about $4 \times 7 \mu$. With the onset of regeneration an increase in the size of the nuclei and nucleoli begins, though it is comparatively slight in the former case.

Twelve hours after a worm is cut such a change in the nucleoli near the wound is apparent and by the second day many of them measure at least $2\ \mu$ in diameter (Fig. 6). Similarly by the second day the nuclei have enlarged until the average of a number measured is $5.1 \times 8.8\ \mu$. This increase in the amount of nuclear and nucleolar materials is not confined merely to the wound region but extends even eleven or twelve segments from it. Farther away than this, however, these structures show no change from the size ordinarily present in such a region (Fig. 5).

Double nucleoli are found in increased numbers within the same limits as are enlarged ones and beyond the twelfth or thirteenth segment from the wound they are no more abundant than in an uninjured individual (Tables I. to VI. inclusive). There is no gradual decrease in number, however, as one gets farther from the wound but a somewhat abrupt drop about the twelfth segment. Mitoses, too, are found only in these same twelve or thirteen segments and, though usually most numerous in the two or three segments nearest the bud, they too do not gradually decrease in number but stop rather abruptly.

TABLE VII.
NUMBER OF DOUBLE NUCLEOLI.

Number of Days of Regeneration.	Segments from Wound.		Total.
	1-5 (incl.).	6-12 (incl.).	
1	40	26	66
2	122	150	272
3	133	127	260
4	183	66	249
5	179	71	250
6	98	52	150

Double nucleoli and mitoses increase rapidly in numbers during the early part of the regeneration period and then gradually decrease again. Double nucleoli have begun to appear on the first day but only one instance of mitosis is found. There is a great increase in the number of each on the second day. By the fourth day, however, the number of double nucleoli has dropped decidedly in the segments more distant from the wound; this is counterbalanced by an increase in the five nearest segments

with the result that the total number is not appreciably changed. Table VII. gives the comparative abundance of double nucleoli in the first five segments and in the other seven which are apparently involved in the regenerative processes.

The number of mitoses also decreases in the more distant portion but in this case it is not compensated by an increase near the wound so that the total number is less. The major part of the activity of the gut in forming new tissue, therefore, is now confined to a more restricted area. Table VIII. gives data for mitoses corresponding to that given in Table VII. for double nucleoli.

TABLE VIII.
NUMBER OF MITOSES.

Number of Days of Regeneration.	Segments from Wound.		Total.
	1-5 (incl.).	6-12 (incl.).	
1	1	0	1
2	32	21	53
3	28	17	45
4	28	5	33
5	25	10	35
6	11	2	13

The records for the fifth day are approximately the same as those for the fourth. On the sixth the numbers both of double nucleoli and of mitoses have decreased decidedly throughout the entire region involved in regeneration. This decrease is most marked, however, in the more distant segments. By the seventh day the intestine of the old region has returned to approximately its usual appearance in an uninjured worm. Only two or three cases of mitosis are to be found and double nucleoli are little if any more abundant than in intact animals.

Beginning with the second or third day many cells in which the nucleoli are small are present in the intestine of the old part. These are probably the products of a recent mitotic division during which the nucleolus has been lost. That the nucleoli of these cells enlarge gradually is evidenced by the fact that the number of such cells does not increase appreciably during further regeneration. Similarly most of the cells in the newly formed gut tissue of the regenerating bud have small nucleoli at first.

By the third day these, too, are enlarging so that it is not possible to discover a line of demarcation between the gut of the bud and that of the old part. At about this time double nucleoli and mitotic figures begin to appear in the new gut. Cell proliferation in the new tissue thus increases as that in the old part is decreasing. Consequently, as far as the intestine is concerned, regeneration usually ceases between the sixth and seventh days. After this time it is a matter of growth in the newly formed tissue itself.

Mesoderm.

The method of formation of the new mesoderm is not as generally agreed upon as is that of the new intestine. Hepke ('97) in *Naids*, von Wagner ('06) in *Lumbriculus* and Abel ('02) in *Tubifex* and *Nais*, among others, maintain that the new mesodermal structures originate from ectodermal cells which migrate into the cœlom. Randolph ('92) and Iwanow ('03) in different species of *Lumbriculus* and Krecker ('10 and '23) in *Tubifex* and *Limnodrilus* find that these structures are derived from relatively unmodified cells of the mesoderm to which the term neoblasts was first applied by Randolph. Krecker ('23) reports that they are ordinarily found on the posterior surfaces of the septa in a quiescent state. After a worm is cut they enlarge to 8 or 10 times their former size and then migrate along the nerve cord to the wound region. According to his data these cells are activated on the seven septa nearest the wound with those in the four nearest segments giving the greatest response. In my preparations of individuals which have been regenerating for either three or four days a number of clearly distinguishable neoblasts are always present as far as eight or nine segments from the wound. Two examples, one of a three-, the other of a four-day regenerate, are given in Table IX. All the cells which lie upon the septa and are clearly neoblasts with nucleoli of $2\ \mu$ or over are included in these counts. All are found on the posterior surfaces of the septa and are confined almost entirely to the ventral portion. These cells correspond presumably to the later stages of metamorphosis described by Krecker ('23). In both of these cases nine segments are clearly contributing neoblasts while the tenth and eleventh are ap-

parently not involved. It is a rather common occurrence to find one or two neoblasts on a septum in an uninjured worm so that the few seen in these last segments are in accordance with what is to be expected. In making these counts, not only leucocytes, as suggested by Krecker ('23), but also nephridial cells are apt to be mistaken for neoblasts in early stages of metamorphosis. Cells of this type, as clearly demonstrated by examination of adjacent sections, are shown in Fig. 9. Both of these types of cells have quite large nuclei and nucleoli and many nephridial cells are of course in contact with the septa.

TABLE IX.
NEOBLASTS.

Segments from the wound.....	1	2	3	4	5	6	7	8	9	10	11
3-day regenerate.....	17	13	16	13	9	6	7	3	6	1	1
4-day regenerate.....	15	19	18	14	8	7	8	8	7	2	1

On the four or five septa nearest the wound, therefore, neoblasts are most abundant, as also observed by Krecker ('23). About nine segments in all, however, seem to be involved while Krecker reports the maximum distance as seven. The region involved in the production of neoblasts is, therefore, approximately the same as that in which cell proliferation occurs in the intestine.

Ectoderm.

The ectoderm, unlike the other two types of tissue already discussed, regenerates solely from cells in the immediate vicinity of the wound. That cells from the old hypodermis form the new hypodermis and nerve cord is generally agreed upon. Krecker ('10, p. 430) describes the marked enlargement of certain ectoderm cells "not directly opposite the nerve but somewhat dorsally, between it and the central longitudinal axis of the body." These cells increase in size and become so changed that, as he expresses it (p. 434), "were these cells seen alone they would be immediately considered neoblasts." Of the changes which take place in cells other than those of this particular region, however, he makes no more than the simple statement (p. 430) that "of course the ectoderm cells are greatly enlarged elsewhere than on the ventral side."

Twelve hours after a worm is cut the wound has healed and in those ectoderm cells which are brought into a terminal position by this process the nucleoli have begun to enlarge (Fig. 10). The cells themselves are beginning to elongate somewhat and accompanying this process the nuclei are changing to the oval shape more or less typical of elongated cells. The nuclei themselves are not appreciably enlarged at this time but the nucleoli have increased from mere dots to quite evident structures about $1\ \mu$ in diameter. Cells of this type are not confined to any definite part but make up the entire terminal portion of the hypodermis. After one day of regeneration the enlargement has gone still further but there is no marked difference in size between cells in the ventral portion and those dorsally located. The average size of the nuclei at this time is $4.5 \times 7.2\ \mu$ and of the nucleoli $1.4\ \mu$.

On the second day, however, characteristic differences make their appearance (Fig. 11). The cells which are dorsal or lateral show little if any change from the preceding day. In the median ventral region just posterior to the end of the nerve cord many of the cells have grown considerably but all gradations in size can be found between the largest of these and the cells found elsewhere in the bud. In the larger cells the nucleoli are oval and about one third the dimensions of the nuclei. The average size of ten of the larger nuclei found in two adjacent sections is $6.1 \times 8.4\ \mu$, with the nucleoli of the same cells averaging $1.9 \times 2.8\ \mu$. On the following day the nuclei of the dorsal cells are of about the same size as on the second day but many of the nucleoli have enlarged to $2\ \mu$. In the ventral region the changes are even more marked. A number of the cells have enlarged enormously and have nuclei of about $8 \times 11\ \mu$, with nucleoli averaging about $3.5\ \mu$. It is this stage of the hypodermal cells to which Krecker applies the term "metamorphosed" ectoderm and which may be most easily confused with neoblasts. Krecker suggests that the fact that the neoblasts come to lie near such cells is probably the reason why so many workers have thought the neoblasts to be products of the ectoderm, which seems very plausible.

Krecker ('10, p. 436) suggests, as a theory of the formation of

these much enlarged cells, that "the neoblasts have a redifferentiating effect upon the cells of the ectoderm." A close examination of the facts, however, demonstrates that the cases which he cites in proof may all be explained in another way.

In the course of regeneration the enlargement of the hypodermal cells does not appear to begin suddenly soon after the arrival of neoblasts at the wound. It seems rather to be a continuous process having its inception at the time the worm is cut. A certain time is required for the cells of the hypodermis to enlarge and those on the ventral side do not change any more rapidly than those elsewhere. They do, however, show a greater response than the others with the result that their enlargement continues after the others have slowed down. In sections of twelve-hour regenerates the ectoderm is quite different from that in uninjured individuals and the process of enlargement is clearly under way. At this time neoblasts are rarely found at the wound. Krecker ('10, p. 422) says that "twelve hours after the operation . . . in one of these (individuals) there was a neoblast at the wound, but none was migrating. In the other individual . . . two were about the wound." If it is true, then, that the enlargement of the ectoderm begins before twelve hours after the cut is made, and it certainly appears to, it is improbable that the neoblasts could have been the cause. A much simpler explanation, which seems to satisfy all of the requirements of the facts at hand, is that whatever is the underlying cause of the metamorphosis of the neoblasts on the septa is likewise the cause of the enlargement of the cells of the hypodermis at the wound. Just what may cause the neoblasts to metamorphose is not discussed by Krecker.

This view that the transformation of the ectoderm cells is independent of the presence of the neoblasts is entirely in accord with the cases cited by Krecker ('10) in proof of his theory. In one individual (p. 433) "even after three days there was no enlargement of the ectoderm. No neoblasts were about the ectoderm, in fact there was only one neoblast to be seen and this was along the nerve some distance away." He says later (p. 436) that this "exception cited in which no change in the character of the ectoderm cells occurred in the absence of the

neoblasts is of considerable significance." This case may be equally well explained in accordance with the view of an independent transformation. The factor which brings about the metamorphosis of the neoblasts and ectoderm is either absent or, because of the physiological condition of this particular individual, produces no effect. It is reasonable to suppose that, if the same underlying cause brings about like changes in these two types of cells and if they are equally susceptible to activation, one type will not undergo a process of transformation when the other does not. In all other cases given by Krecker for posterior regenerates he himself states (p. 435) that "the evidence adduced has to do only with instances in which neoblasts are found about the metamorphosed ectoderm cells." Admittedly neoblasts are present in all cases where the ectoderm cells are greatly enlarged, but an explanation for this is easily found. Neoblasts migrate only along the ventral nerve cord and so they are found in the ventral region of the bud. The ectoderm cells which are greatly enlarged are those which are to give rise to the nerve cord in the ventral region. The close proximity of these two types of cells in the regenerating bud seems to be due to the fact that both bear a definite relation to the nerve cord—those of the hypodermis to form the new portion of the nerve cord in the bud and the neoblasts using it as a pathway to the wound region. In the discussion of anterior regeneration his statement that no metamorphosis of the ectoderm and no migration of the neoblasts occurs in this type of regeneration will be considered.

The development of the setigerous glands and the production of the setæ have for the most part been neglected in the work on regeneration. Bergh ('90) and Penners ('23) both give something of a description of their formation during embryonic development. In regeneration it is difficult to observe the early stages due to the great number of cells scattered throughout the growing bud. By the fifth day the cells of the glands in the two or three segments of the bud nearest the old tissue stand out clearly since at this time the other cells of these segments have become arranged in a more orderly fashion. The nuclei of the hypodermal cells near the old tissue have begun to take on the appearance typical of the older cells. They are

oval—measuring about $3.5 \times 6 \mu$ —and contain nucleoli less than 1μ in diameter. The nuclei of these gland cells, however, have not decreased in size but are about the same as those of the enlarged ectoderm cells of the dorsal and lateral regions (Fig. 12). The average measurement of twenty-five of these is $5.2 \times 7.3 \mu$ with nucleoli 1.8μ in diameter. Occasionally double nucleoli are to be found. The fact that these cells push into the coelom offers another point of confusion which may have led early workers to think that the ectoderm migrated into the coelom to form the new mesodermal structures.

It will be seen that in all three types of cells (endodermal, mesodermal and ectodermal) which take part in the formation of new tissue at the posterior end there is one outstanding feature in common. The nuclei and nucleoli enlarge. The greatest changes occur in the neoblasts and in the cells of the ventral portion of the hypodermis. In both of these types the nuclei of the fully transformed cells are eight or nine times as large as ordinarily. The nucleoli increase even more in proportion, particularly in the hypodermal cells in which they enlarge from mere dots to structures over 3μ in diameter. Even in the gut cells where the nucleoli are usually of considerable size there is probably a ten-fold increase. The significance of these facts will be discussed later.

ORIGIN OF NEW TISSUE IN ANTERIOR REGENERATION.

Endoderm.

In anterior regeneration as in posterior regeneration the majority of investigators—Rievel ('96), Haase ('98), von Wagner ('00), Iwanow ('03) and Krecker ('10)—are agreed that most of the intestine of the regenerating bud is formed by the growth of that in the old part. This formation of new tissue involves about eleven or twelve segments here as in posterior regeneration. The nucleoli are considerably enlarged at the end of the first day and double nucleoli are fairly common. Only a few mitoses, however, are present. The numbers both of double nucleoli and of mitoses reach a maximum between the second and third days. On the fourth day both are fewer in number in the more distant segments. In the case of double nucleoli this decrease is partially

compensated by an increase in the region nearest the wound. Mitoses, however, are somewhat less frequent in this region too. As in posterior regeneration the numbers continue to decrease and by the seventh day both of these features are rare in the gut of the old part. Tables X. and XI. give records for two- and four-day regenerates, respectively. Tables XII. and XIII. give comparative data for the nearer and more distant segments for double nucleoli and mitoses, respectively.

There is little, if any difference, then, in the behavior of the cells of the intestine in anterior and in posterior regeneration.

TABLE X.
2 DAYS OF REGENERATION.

Segments from the wound.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli.....	27	26	21	27	34	30	30	33	32	29	16	16	1	1
Mitoses.....	12	6	3	7	8	4	3	4	2	3	2	0	1	0

TABLE XI.
4 DAYS OF REGENERATION.

Segments from the wound.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli.....	38	31	29	32	34	26	14	7	8	6	2	3	3	1
Mitoses.....	12	4	3	4	7	3	1	0	1	1	0	0	1	0

TABLE XII.
DOUBLE NUCLEOLI.

Days of Regeneration.	Segments from Wound.		Total.
	1-5 (incl.).	6-12 (incl.).	
2	135	186	321
4	164	66	230

TABLE XIII.
MITOSES.

Days of Regeneration.	Segments from Wound.		Total.
	1-5 (incl.).	6-12 (incl.).	
2	36	18	54
4	30	7	37

Mesoderm.

Many investigators—Hepke ('97), von Wagner ('00) and Abel ('02) among others—are of the opinion that the mesoderm both of anterior and of posterior regenerates is formed from the ectoderm. The weight of evidence in posterior regeneration, as previously mentioned, now seems to favor mesoderm formation from neoblasts. In anterior regeneration Iwanow ('03) in *Lumbriculus variegatus* and Krecker ('10) in *Tubifex* and *Limnodrilus* find that the new mesoderm is formed from the old mesoderm and not from regeneration cells as in posterior regeneration. They find a distinct fraying out of the longitudinal muscles at the wound. Many of the cells then lose their contractile substance and wander about in the cœlom where they become mixed with other cells—peritoneal, connective tissue and ectodermal—so that it is "hard to distinguish between the various types" (Krecker, '10, p. 441). After about the fifth day of regeneration they begin to develop contractile substance and to become arranged in muscle masses. The production of the body musculature is much slower in anterior than in posterior regeneration. "In specimens killed three weeks after the operation the musculature of this region was still in a very undeveloped state" (Krecker, '10, p. 442).

As the matter stands at present, then, investigators are divided into two groups regarding the formation of the new mesoderm. Those in one believe that it is derived from the ectoderm, those in the other that it is produced by a partial dedifferentiation followed by a redifferentiation of the cells of the old mesodermal structures. Those who support an ectodermal origin of the mesodermal structures believe that the mesoderm is formed in the same manner at both ends. Those of the other group, however, think that the origin is due to one type of mesodermal cell at the posterior end—the neoblasts—and to another at the anterior end—the specialized cells which dedifferentiate.

In anterior regeneration, just as in posterior regeneration, there are many opportunities for confusion. In the former it is even more difficult than in the latter to follow just what happens. The migration of ectoderm cells into the cœlom in the formation of the cerebral ganglion might easily mislead one. In addition

to this the presence of many other cells of similar appearance make it practically impossible to say, from an examination of fixed specimens, just what cells do form the various tissues. Since in posterior regeneration it is quite apparent that ectoderm is not involved in the formation of the septa or longitudinal muscles it seems reasonable to assume that it does not take part here. This view is supported further by the fact that cells of mesodermal origin are present in the bud.

Regarding the conception that there is a partial dedifferentiation of the old muscle cells which then form the longitudinal musculature of the bud, there seems to be conflicting evidence. These cells certainly do fray out and some of them seem to lose their contractile substance in the segment injured by the cut. Such a behavior is not, however, peculiar to the anterior end in *Lumbriculus inconstans*. Just as other cells are affected by the cut so many muscle cells are dislodged or injured. It is not improbable that such cells should then lose their contractile substance due to the injury; such a change might, therefore, be a step on the road to destruction rather than on that to repair. The fact that this same sort of change does take place at a posterior cut-surface, where the muscle cells are not involved in the regenerative processes, seems to support this view. The migrating spindle-shaped cells, which appear between the first and second days in *Lumbriculus*, are clearly derived from the hypodermis (Fig. 13). These cells migrate into the coelom and there form the cerebral ganglion. It may perhaps be this type of cell which has been observed by Krecker in *Tubifex* and *Limnodrilus*.

Further evidence is derived from a study of neoblasts in anterior regenerates. Krecker finds that in the species with which he worked they are usually not activated at all posterior to a cut and that resting neoblasts may be found on a nearby septum. "The individuals upon which these observations were made were all killed three weeks or more after the operation so that the failure of the neoblasts to act as they do at the posterior end could hardly have been due to lack of time" (Krecker, '10, p. 437). In the individuals used in his experiments—of the genera *Tubifex* and *Limnodrilus*—anterior regeneration does not

take place at the level of the twentieth somite where these cuts were made. It does not seem strange, therefore, that at the end of three weeks all neoblasts should be resting even if activation had taken place three weeks previous. In *Lumbriculus* anterior regeneration does take place at all except very posterior levels. The amount of tissue is, of course, limited to a few segments, usually five or six, rarely seven (von Wagner, '00).

Counts made of the number of clearly recognizable neoblasts (intermediate and definitive stages of Krecker, '23) present on the various septa in three- and four-day regenerates are given in Table XIV. These cells all possess nucleoli of approximately $2\ \mu$ or over (Fig. 14).

TABLE XIV.

NEOBLASTS.

Segment from the wound.....	1	2	3	4	5	6	7	8	9	10	11
3-day regenerate.....	6	21	14	9	11	13	3	3	4	2	1
4-day regenerate.....	16	14	23	13	7	8	2	6	3	1	1

These figures do not differ greatly from those for posterior regenerates (Table IX.). The small number of neoblasts present on the first septum of the three-day regenerate may be explained in part by the fact that this septum was partially torn away by the cut in this particular case. About four segments seem to produce neoblasts more abundantly than the rest and about nine segments in all are apparently involved. The more active region extends farther, however, in the three-day regenerate; the inclusion of the first septum in the cut brings this about. The neoblasts, then, are activated to about the same extent as at the posterior end. The number present in the bud at the end of two days, however, indicates that either not as many migrate anteriorly or else the migration is much slower and the cells are used up as they get to the wound. No mass of large cells is to be found in the ventral part but several cells are usually present and a number can generally be found migrating along the nerve cord (Fig. 15). The apparently slow migration of the neoblasts anteriorly is perhaps the reason why the mesoderm in the newly formed head does not show signs of development until after the

fifth day (Krecker, '10). After the third day it is difficult to say just what does happen in the bud region. Many metamorphosed ectoderm cells are present and things are so confused that it is difficult to distinguish the various types of cells, particularly those as similar in appearance as are the neoblasts and the much enlarged cells from the ventral hypodermis.

The process of formation of new septa and longitudinal muscles by cells known to be derived from neoblasts has not been observed. The fact that neoblasts metamorphose in considerable numbers and are frequently seen migrating along the nerve cord is, nevertheless, evidence which seems to indicate that they play some part in anterior regeneration in *Lumbriculus*. If such is the case, the mesodermal structures are formed in the same manner in both types of regeneration, just as are the ectoderm and endoderm.

Ectoderm.

As in posterior regeneration only those cells in the immediate vicinity of the wound take part in the formation of the new ectodermal structures. There occurs a similar enlargement of all these cells during the first day or so, no one region undergoing any more extensive change than any other. Iwanow ('03) in his discussion of the formation of the new nerve elements at the anterior end describes a marked enlargement of the ectoderm cells. Krecker ('10, p. 433) says that the ectoderm cells "undergo no such metamorphosis" as at the posterior end, explaining this on a basis that no neoblasts migrate anteriorly to stimulate it to activity. As in the case of posterior regeneration, however, by the second day there are cells on the ventral side which have quite large nuclei and nucleoli, and by the third day they have reached a considerable size (Fig. 16). The area over which this marked enlargement extends is greater than in posterior regeneration. Large nuclei are present in cells found over a considerable portion of the ventral and ventro-lateral hypodermis and even somewhat dorsally in the terminal portion. The largest cells, nevertheless, are confined for the most part to the ectoderm in the vicinity of the mid-ventral line. This region extends from the end of the old nerve cord anteriorly. The nuclei and nucleoli measure about the same as in the transformed cells of the

regenerating tail ($8 \times 11 \mu$ and 3.5μ , respectively). In the dorsal portion of the terminal hypodermis many of the cells are apparently in the process of breaking away from the epithelium to migrate into the coelom where they form the cerebral ganglion (Fig. 13). They become elongated and slender in this process and develop an appearance which answers well the description of cells which Krecker ('10, p. 441) believes are mesoderm cells migrating anteriorly to form the new longitudinal muscles—"spindle-shaped cells with slightly granular cytoplasm and large nucleus containing a deeply staining nucleolus."

Regarding the activation of the ectoderm by the presence of the neoblasts, there is no further evidence from anterior regeneration. Neoblasts apparently migrate anteriorly and the ectoderm is also transformed, similar processes to those occurring at the posterior end.

From these observations it is apparent that not only the ectodermal and endodermal elements of the regenerated head are derived in the same manner as at the posterior end but the mesodermal structures as well. Just as in posterior regeneration the neoblasts apparently metamorphose and migrate to the wound region and the cells of the ventral portion of the hypodermis become greatly enlarged. About ten or eleven segments seem to be involved in the regenerative processes except in the case of the ectoderm in which the changes are confined to the immediate vicinity of the wound as in posterior regeneration.

DISCUSSION.

The production of new tissue in the anterior regeneration of microdrilous annelids seems to be essentially the same as in the posterior regeneration in these forms. Iwanow ('03) in *Lumbriculus variegatus* and Krecker ('10) in *Tubifex* and *Limnodrilus* describe the formation of the new mesodermal tissue from neoblasts at the posterior end but believe that there is a dedifferentiation of old mesoderm to form the new at the anterior end. The loss of contractile substance by some of the longitudinal muscle cells at the cut surface, however, occurs at both ends. The spindle-shaped cells abundant in the dorsal part of the bud in anterior regeneration may be seen in *Lumbriculus inconstans*

to be derived from the hypodermis, migrating into the cœlom to form the cerebral ganglion (p. 295). The fact that many neoblasts metamorphose and migrate anteriorly is evidence that they probably take part in the building of the new mesoderm in anterior as well as in posterior regeneration. The presence in the bud of other cells of similar appearance makes it practically impossible to follow the laying down of these structures from cells of known origin. It has for some time been agreed by investigators that both endodermal and ectodermal structures are formed in the same manner in both types of regeneration, that is, from the old intestine and body wall, respectively. It seems evident then that the mesodermal structures are produced in the same way at both ends and are not an exception as previously believed.

An explanation for the formation of the new mesoderm from more or less undifferentiated mesodermal cells rather than from the muscles may perhaps be found in the fact that the cytoplasm of the muscle cells is highly modified. While the cells of the hypodermis and intestine are of a simple, cuboidal or columnar shape, the development of the contractile substance by the muscle cells brings about an extensive modification of the cytoplasm of these cells. Consequently, cells from the peritoneum, less modified than the others are called upon to form the tissue in the regenerating bud. The development of the new nerve cord is a process not essentially different. In this case the cytoplasm is considerably modified in the formation of fibers and, instead of the nerve cord near the wound dedifferentiating to produce the new tissue, the hypodermal cells of the ventral side are called upon to furnish the new material. While these facts may not necessarily indicate that one type of cell is any "more differentiated" than another, the muscle and nerve cells in *Lumbriculus* certainly are less susceptible to the activating stimulus than are those of the other tissues.

In regeneration the ectoderm cells enlarge considerably especially those in the median ventral line which may develop nuclei as large as $8 \times 11 \mu$ with nucleoli between 3 and 4μ in diameter. Krecker ('10 and '23) is of the opinion that this change in the ventral cells is due to "some influence" of the

neoblasts. Evidence presented here (pp. 290 and 297) does not support this idea but rather the view that a similar underlying cause brings about the changes which occur in the metamorphosis both of the neoblasts and of the ectoderm cells. During the first two days of regeneration all the cells of the hypodermis in the immediate vicinity of the wound develop large nuclei and nucleoli. After this time the ones found dorsally and laterally slow down whereas those in the ventral region continue to enlarge. Neoblasts are rarely, if ever, found at the wound at the time when the increase in size of the hypodermal cells becomes apparent (between six and twelve hours after the cut). From that time until the largest cells are formed the enlargement seems to be a continuous process without any sudden change or increase in rate which might be produced by the presence of neoblasts. The fact that in posterior regeneration metamorphosed ectoderm cells and neoblasts are always found very near one another is cited by Krecker ('10) as proof that there is some relation between them. This proximity of these two types of cells is perhaps equally well explained if the reason for the presence of each in the ventral region is considered. The cells of the ventral hypodermis are transformed in the process of proliferation and migration to form the nerve cord of the regenerating region. The neoblasts migrate to the posterior end from the old tissue along this same structure. These cells, then, are brought together not because one causes the modification of the other, but rather from the fact that each type has a certain relation to the nerve cord. The view that the same cause produces the changes in the cells of both types seems to fulfill all the requirements and to be a simpler explanation than that given by Krecker. Furthermore, there is no apparent reason why the ectoderm, any more than the endoderm, should need to be activated by another type of cell.

Function of the Nucleolus.

Various theories have been advanced regarding the function of the true nucleolus or plasmosome. Montgomery ('98) and Ludford ('22) give a rather complete discussion of the work of many investigators. The more recent results seem to indicate

that the nucleolus bears some relation to the activity of the cell body and nucleus. Many cases have been reported of nucleolar extrusions preceding the formation of yolk granules. Ludford ('22) also reports that in the more active cells of the endoderm of *Limnaea* the nucleoli are larger than in those of the hypodermis. He is "inclined, therefore, to regard the size of the nucleolus as an indication of the degree of metabolism existing in the cells—the greater the metabolic activity, the larger the total volume of nuclear (nucleolar ?) matter present in the nucleus, or extruded into the cytoplasm" (p. 139). Wilson ('25, p. 96) also believes that there is a "question whether the nucleolus may not play a more active and important part in cell metabolism than most writers have hitherto assumed."

An examination of slides of *Lumbriculus*, both of uninjured and of regenerating individuals reveals no cases of nucleoli which could be interpreted as being extruded into the cytoplasm from the nucleus. There is considerable evidence, however, that nucleolar size is in some manner an indication of the degree of metabolic activity of the cell.

In uninjured worms the gut cells of the first eleven or twelve segments have small nucleoli. Similarly those for some distance from the anal opening have a comparatively small amount of nucleolar substance. In the intermediate portion, however, relatively large nucleoli are present. The cells of the mouth cavity and esophagus naturally do not take as great a part in the digestive processes as do those found more posteriorly. In the mid-gut the digestive fluids are being poured into the lumen and the food is being absorbed. Consequently considerable cell activity is necessary. Toward the posterior end such activity naturally drops off again. The size of the nucleoli, therefore, parallels more or less the extent of the activity expected of the cells in the various regions of the gut (p. 281).

Furthermore, in the case of the setigerous glands, the nucleoli of the cells are large in the growing tail region and in a regenerating bud where the new setæ are being rapidly formed (pp. 281 and 291). In the old segments of a worm, however, where the setæ have been present for a considerable time, the nucleoli are usually small (p. 281). The cells in the active

portion of the nephridia possess nucleoli of considerable size, too. In fact, these cells are very similar in appearance to intermediate neoblasts, from which they may be distinguished by the presence of large granules in their cytoplasm (p. 288). Similarly, as described by Krecker ('23), the phagocytes have very large nucleoli.

Again, in the growing tail, just as in the regenerating individual, the hypodermal cells have enlarged nucleoli, particularly on the ventral side where the nerve cord is being formed (p. 280). Those cells which are forming the new mesoderm—presumably derived from the primary mesoblasts in the embryonic development (Wilson, '89 and '92)—also have very large nucleoli, differing very little in appearance from the neoblasts in a regenerating tail (Randolph, '92). In contrast to this, in the older part of the worm, the nucleoli of the mesoderm and ectoderm cells are very small, in the case of the latter mere dots under a magnification of 1,000 \times .

In regeneration, as already mentioned, the nucleoli of the neoblasts and hypodermal cells become greatly enlarged. There is also at this time an increase in the amount of nucleolar substance in the gut cells. For ten or eleven segments from the wound, the nucleoli enlarge, a process followed by the appearance of numerous instances of double nucleoli—two within a single nucleus (p. 285).

This occurrence of double nucleoli is taken by Iwanow ('03) and Krecker ('10) as evidence that amitosis is frequent in the production of the new gut tissue. In *Lumbriculus* there is no evidence of any division or even of a clearly defined constriction in any of the nuclei of the gut which contain two nucleoli. The individual nucleoli in the case of the double one are usually smaller, and in no case larger, than those in the neighboring cells where but a single nucleolus is present. It seems, then, that the division of the nucleolar material into two parts is not in preparation for a succeeding cell division. Rather as this material accumulates it continues to exist in a single droplet until it reaches a certain size and then divides. This splitting into two parts may perhaps be due to the fact that a droplet of material of its consistency and composition has a certain

maximum size beyond which it cannot exist as an individual droplet under the conditions existing in the nucleus. This view is in accord with our knowledge of the limitations to drop size in emulsions.

After one day of regeneration many cells show this increase in the amount of nucleolar material, as evidenced by the occurrence of a number of double nucleoli as well as the enlargement of the single ones. On the second and third days the frequency of double nucleoli reaches a maximum and after that time falls off slowly. Following the increase in nucleolar material, there appear numerous cases of mitosis. It seems probable that this increase in nucleolar substance is indicative of a heightened activity on the part of the cells in preparation for cell division.

A comparison of the amount of nucleolar material in the various types of cells taking part in regeneration reveals a distinct parallelism between this amount and the relative activities of these cells. There are four general types of cells involved: (1) those of the old gut which form the new gut; (2) those of the old dorsal and lateral hypodermis which build the new hypodermis; (3) those of the old ventral hypodermis of which the special function is to furnish the material for the nerve cord in the regenerating bud; finally (4) the neoblasts which form the new mesodermal structures. Of these, the cells of the first two types maintain to a certain extent their usual epithelial arrangement, only a comparatively small amount of migratory activity occurring. Their nucleoli enlarge considerably but by no means as much as in the case of the other two types. The ventral ectoderm cells and the neoblasts, when fully transformed, are of about the same general size and appearance and have nucleoli of nearly twice the diameter of those in the other cells. The ventral ectoderm cells must naturally undergo rapid proliferation to supply all of the material necessary for the nerve cord; the neoblasts must migrate to the wound region and there multiply with considerable rapidity. There is some relation in *Lumbriculus*, then, between the functional activity of the cells and the amount of nucleolar material present in them. This is a conclusion similar to that drawn by Ludford ('22) from a study of *Limnæa* with particular reference to the behavior of the nucleolus in oogenesis and cleavage.

SUMMARY.

Nuclei and Nucleoli in Uninjured Individuals.

1. The nuclei and nucleoli of the hypodermal cells are small except in the growing tail region. Here they are enlarged, especially in the cells on the ventral side which are involved in the formation of the new nerve cord.
2. Large nucleoli are present in the cells of the setigerous glands near the growing region of the tail. In old segments they are small.
3. The gut nucleoli are small in the first twelve segments. They are larger from this region up to twenty or thirty segments from the posterior end. In these segments, they are again small.
4. Double nucleoli are occasionally found in the mid-gut, where large nucleoli are present.

Origin of New Tissue in Regeneration.

5. Double nucleoli and mitoses are found in the intestine for eleven or twelve segments from the wound. In this same region the nucleoli are considerably enlarged.
6. Cell proliferation in the old intestine practically ceases between the sixth and seventh days of regeneration.
7. Neoblasts metamorphose and migrate to the wound at the anterior end as well as at the posterior end. At least eight or nine segments furnish these cells, the four or five nearest the wound apparently playing the most important part as observed by Krecker.
8. The failure of the muscle and nerve cells of the old part to form the corresponding new structures in regeneration is perhaps due to the fact that the cytoplasm of these cells has become highly modified, thus rendering them less susceptible to activation.
9. The spindle-shaped cells in the dorsal portion of the bud cavity at the anterior end are derived from the hypodermis and not from the muscles of the old part.
10. In both anterior and posterior regeneration the nuclei and nucleoli increase in size in the ectoderm cells in the immediate

vicinity of the wound. This enlargement is no more rapid in one part than in another; it continues longer in the ventral cells so that by the second day it is greater there.

11. The metamorphosis of the ectoderm is not in all probability due to the proximity of the neoblasts, as supposed by Krecker, but instead to an independent transformation.

12. The cells of the setigerous glands in the new bud have large nuclei and nucleoli.

13. A feature common to all the cells which take part in the formation of the tissues in the regenerating bud is the presence of large nuclei and nucleoli.

14. The amount of nucleolar material present in a cell seems to be an index of the activity of its nucleus both in cell-metabolism and in preparation for cell division.

15. In *Lumbriculus* there is no evidence of any division or even of a clearly defined constriction in any of the nuclei of the gut which contain two nucleoli.

16. The presence of two nucleoli in a single nucleus is not a step in amitosis, as many have supposed, but is due to the increase in nucleolar substance beyond the amount which can exist within that particular nucleus as a single droplet.

17. The various tissues seem to be derived in the same manner both in anterior and in posterior regeneration.

CONCLUSIONS.

1. Both in anterior and in posterior regeneration the mesoderm is formed from neoblasts. Iwanow and Krecker are in error in the belief that cells from old specialized mesodermal structures form the new ones in the anterior regeneration of *Lumbriculus*.

2. There is a certain predetermined area of the hypodermis on the ventral side which metamorphoses preparatory to the formation of new nervous tissue during regeneration. The cells of this region are probably activated by the same stimulus as are the neoblasts. Krecker's view that the neoblasts have an inciting effect on the cells of this region seems unfounded.

3. The amount of nucleolar material present in a cell seems to be an index of the activity of its nucleus both in cell-metabolism and in preparation for cell division. Two nucleoli within a single

nucleus are the result of an increase in nucleolar substance beyond the amount which can exist within the nucleus as a single droplet. Their presence is not a step in amitosis.

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KEY TO PLATES.

All figures are photomicrographs. With the exception of Fig. 11, all were taken with a Zeiss apochr. 2 mm., eyepiece 10; these are magnified 640 X. For Fig. 11, a B. and L. apochr. 4 mm. and eyepiece 6 were used; this is magnified 290 X. The following are the symbols used in the figures:

<i>a.</i> ., anal opening,	<i>m. e.</i> ., metamorphosed ectoderm cells,
<i>c.</i> ., cuticle,	<i>mes.</i> ., mesoblast,
<i>chl.</i> ., chloragogue cells,	<i>migr.</i> ., migrating ectoderm cell,
<i>d. e.</i> ., enlarged dorsal ectoderm cells,	<i>n.</i> ., nucleus containing nucleolus,
<i>d. n.</i> ., double nucleoli,	<i>neph.</i> ., nephridial cell,
<i>g.</i> ., gut,	<i>nb.</i> ., neoblast,
<i>gl.</i> ., gland,	<i>nv.</i> ., nerve cord,
<i>h.</i> ., hypodermis,	<i>s.</i> ., seta,
<i>m.</i> ., mitosis,	<i>sep.</i> ., septum.
<i>m. c.</i> ., mouth cavity.	



PLATE I.

Uninjured Individuals.

FIG. 1. Mouth cavity and hypodermis, showing small nuclei and nucleoli in this region.

FIG. 2. Growing region at posterior end.

FIG. 3. Setigerous gland of an old segment.

FIG. 4. Gut of fourth segment.

FIG. 5. Gut of thirteenth segment.



Fig. 1.



Fig. 5.

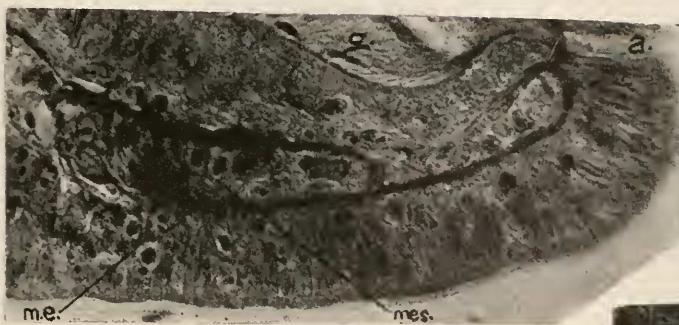


Fig. 2.



Fig. 3.



Fig. 4.

PLATE II.

Regenerates.

FIG. 6. Two-day posterior regenerate. Gut cells of second segment from wound.

FIG. 7. Same individual as in Fig. 6. Gut cells of tenth segment from wound.

FIG. 8. Same individual as in Fig. 6. Double nucleoli in gut cells of fourth segment from the wound.

FIG. 9. Three-day anterior regenerate. Nephridial cells in a position in which they might be mistaken for neoblasts. Arrow points toward the anterior end.

FIG. 10. Twelve-hour posterior regenerate. Enlarging ectodermal cells in wound region.

FIG. 11. Posterior regenerating bud at the end of two days.



Fig. 6.



Fig. 7.



Fig. 10.

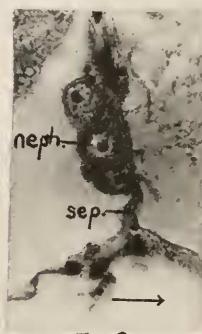


Fig. 9.

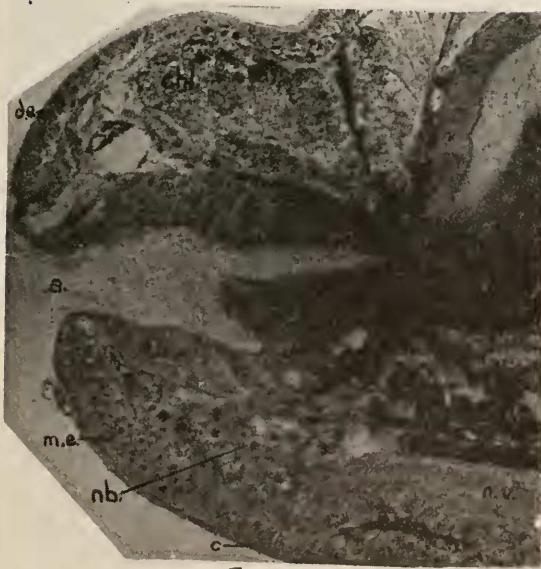


Fig. 11.



Fig. 8.

PLATE III.

Regenerates.

FIG. 12. Six-day posterior regenerate. Setigerous glands producing new setæ.

FIG. 13. Two-day anterior regenerate. Spindle-shaped cells of ectoderm migrating into bud cavity.

FIG. 14. Two-day anterior regenerate. Neoblasts metamorphosing on the posterior surface of the fifth septum from the wound. Arrow points toward anterior end.

FIG. 15. Two-day anterior regenerate. Neoblasts at anterior end of nerve cord.

FIG. 16. Three-day anterior regenerate. Metamorphosed cells of ventral ectoderm.



Fig. 12.

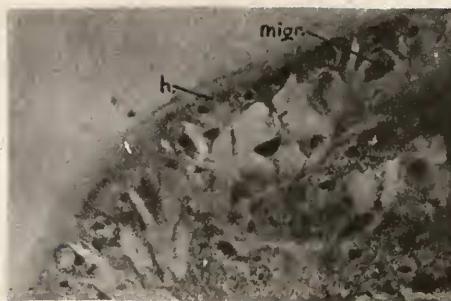


Fig. 13.



Fig. 14.



Fig. 15.

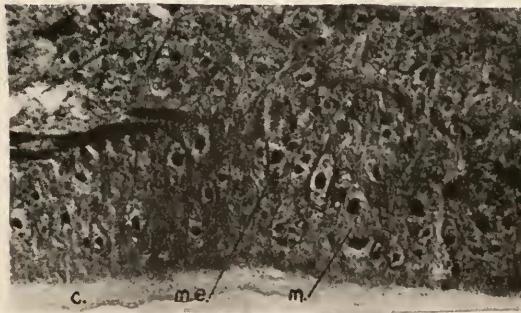


Fig. 16.

BIOLOGICAL BULLETIN

THE EFFECT OF CYANIDES ON THE SWELLING OF PROTOPLASM.

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Physiologists have long since attempted to formulate a theory for the cause of anaesthesia, and the results thereof have been many and varied. The exceptions to any one theory are too numerous and too important to accept any one of them as the correct theory. The final and real solution of so complex a physiological process will probably be one which will take into consideration portions of all theories now existing, or one which will introduce an entirely new factor or factors. Accounts of the various existing theories are set forth in a number of papers among which are the following: Overton ('01), Meyer ('99), Traube ('19), Warburg ('14), Mathews ('14), and Lillie ('18). This paper will make no attempt to give any evidence in support of any one of the theories, but will rather present the results obtained in using HCN and KCN as anaesthetics.

The work here reported deals with the effects of HCN and KCN on the permeability of unfertilized *Arbacia* eggs to water. An increase in the volume of the eggs, when placed in a solution, is taken as showing an increase in the permeability of the egg. Heilbrunn ('25), however, objects to calling an increase in the volume of the egg an increase in permeability. He suggests that this increase in size may be due to a decrease in surface tension; an increase in the fluidity of the interior or an increase in the extensibility of the plasma membrane. Since *Arbacia* eggs are nearly all spherical, changes in their volume may be measured by

measuring the changes in the diameters of the eggs with an ocular micrometer. Eggs from a single individual were placed in concentrations of HCN varying from $N/300$ to $N/2,000$. At the end of ten minutes and again after 25 and 66 minutes, eggs were pipetted into a 50 per cent. solution of sea-water (hypotonic) and their diameters measured at regular time intervals. A control was kept in which sea-water replaced the HCN solutions.

The HCN solutions were prepared by drawing over, by means of an aspirator, into a wash bottle containing distilled water, the volatile HCN gas from another bottle containing KCN to which a few drops of acid had been added. The amount of HCN going into solution was determined by titrating with 0.1 N AgNO_3 ; 1.0 cc. of the nitrate being equal to 0.013 gram HCN. It was necessary to use distilled water because the chlorides in the sea-water interfered with the titration. The desired concentrations of HCN were then made up with sea-water. All the sea-water mentioned in these experiments was sea-water from which CO_2 , in excess of that in equilibrium with the CO_2 in the air, had been removed. This was done by adding 2.4 cc. of 0.1 N HCl per liter of sea-water and aerating for 18 to 24 hours. NaOH or HCl was then added to bring it to the pH of normal sea-water.

The eggs, after exposure to the cyanide solutions, were placed in syracuse watch-glasses containing the hypotonic solution and a 4 mm. objective used as a water immersion with a 10 \times ocular. This gave a magnification of 450 \times . Readings were taken one minute after placing in the hypotonic solution and thereafter every minute for ten or fifteen minutes. A stop watch was used to read time intervals. The following abstract taken from daily notes will show the routine followed throughout the experiments.

7/20/26. Temperature of room 21° C. pH of HCN solution measured colorimetrically = 7.2. 9A.M. unfertilized *Arbacia* eggs placed in 30 cc. of $N/300$ HCN in a finger bowl and covered. 9.10 A.M. eggs pipetted into 5 cc. hypotonic sea-water in watch glass and diameter of eggs measured every minute for ten minutes. Three perfectly spherical eggs were measured each time and the average taken. 9.25 A.M. some eggs from original $N/300$ HCN solution placed in hypotonic solution and ten one-minute readings again taken. Temperature of room 21.2° C. 10.06 A.M., after

exposure of 66 minutes to HCN solution, ten one minute readings again taken. Temperature of room 21.7° C.

The 10, 25 and 66 minute readings were repeated using solutions of HCN up to $N/2,000$. Similar readings were also taken using sea-water instead of HCN solutions. These served as controls. Each time that a new sea-urchin was used, the diameters of the eggs were first measured to be sure that they were approximately the same size as eggs which had been previously used.

As previously stated, the HCN was drawn over into distilled water and then diluted with sea-water. The original HCN solution (with distilled water) usually reached its saturation point as an $N/130$ HCN solution. In order to bring this to an $N/300$ HCN, an amount of sea-water almost equal to the original amount of distilled water had to be added. This in itself, therefore, was a 57 per cent. solution of sea-water and the change in volume of the eggs might very well be due to that hypotonicity instead of to the effect of the HCN. This was checked by adding to the control solutions of sea-water, as much distilled water as was contained in the various concentrations of HCN. That is, the control for the $N/300$ HCN was a 57 per cent. sea-water solution; for the $N/500$ HCN a 74 per cent. sea-water and for the $N/2,000$ HCN a 93 per cent. sea-water solution. The results with these various controls showed that only in the high dilutions, *i.e.*, 57 per cent., 67 per cent. and 74 per cent. sea-water solutions, did this hypotonicity have any appreciable effect on the volume of the eggs, but even this increase was much less than the increase in volume of the eggs previously exposed to the HCN solution. The dotted curve in Fig. 1 marked Control A is the control in 100 per cent. sea-water, while the curve marked Control B is the control in 67 per cent sea-water (comparable to the $N/400$ HCN). The controls for the other concentrations have been omitted to avoid confusion, but in every case the volumes of the eggs in the controls were less than the volumes in the respective HCN solutions. In other words, the volumes of the eggs in the HCN solutions as shown in Fig. 1 are slightly greater than they would be if the HCN solutions had been entirely made up with sea-water. Since the KCN solutions were made up with sea-water only, these precautions were unnecessary for that series of experiments.

In order to test the narcotic properties of the cyanides, eggs were inseminated and after three minutes, placed in progressive dilutions of KCN and HCN. An $N/100,000$ HCN solution still inhibited cell division, while an $N/30,000$ KCN solution was the lowest concentration which would inhibit cell division. Eggs which had been inseminated and then narcotized, were replaced in sea-water and the time for first cell cleavage to appear noted. Eggs which had been exposed to various concentrations of both HCN and KCN for varying lengths of time, were then washed by letting them fall to the bottom of a test tube filled with sea-water and then transferred to sea-water in a watch-glass. These were then inseminated to see whether the eggs were still alive after the effects of the cyanides and the hypotonic sea-water.

The effect of KCN on the permeability was followed merely for comparison with the HCN and ten one-minute readings were taken of eggs in 50 per cent sea-water which had previously been exposed for 25 minutes to concentrations of KCN varying from $N/300$ to $N/900$. The KCN solutions were made up entirely with sea-water.

RESULTS.

Exposure of *Arbacia* eggs to HCN causes an increase in the volume of the eggs when placed in hypotonic sea-water, above that of the controls exposed to sea-water. The volume of the eggs varied directly as the concentration of the HCN and as the time of exposure to the HCN solutions. As previously stated, the fact that the HCN solutions were in themselves hypotonic in varying degrees, did not interfere greatly with the final results, since the increase in volume due to this hypotonicity was only slight as compared to the increase in volume due to the HCN. All eggs exposed to the HCN and to the sea-water controls finally reached the same equilibrium point, $4.518 \times 10^2 \mu^3$. Only eggs which had approximately the same size at the beginning of the experiments, $2.381 \times 10^2 \mu^3$, were used. The effect of the HCN was to hasten reaching the equilibrium point. Fig. 1, in which the volumes of the eggs after definite exposures to HCN are plotted against the time in the hypotonic sea-water, shows the rate at which the increase in volume occurs. Fig. 2 shows the

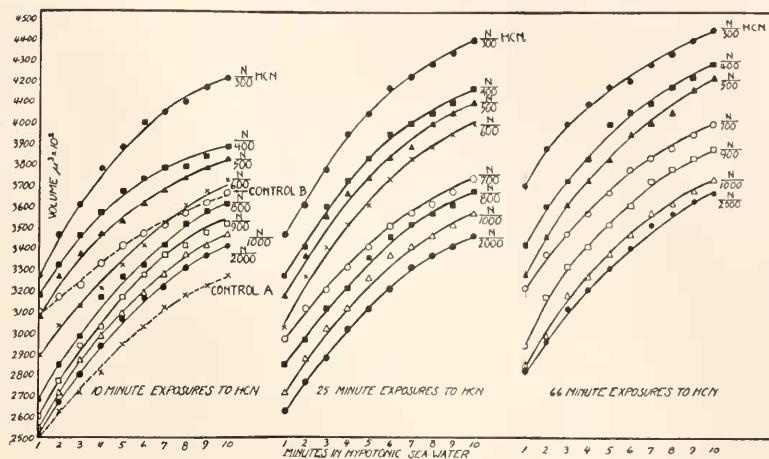


FIG. 1. Volume of eggs after definite exposures to various concentrations of HCN plotted against the time in 50 per cent. sea-water, showing the rate of increase in volume. Control A was exposed to 100 per cent. sea-water instead of HCN while control B was exposed to 67 per cent. sea-water, comparable to the hypotonicity of the $N/400$ HCN. Controls in other percentages of sea-water comparable to the remaining concentrations of HCN have been omitted to avoid confusion, but in all cases, the volumes of the eggs in sea-water were less than the volumes in the HCN solutions.

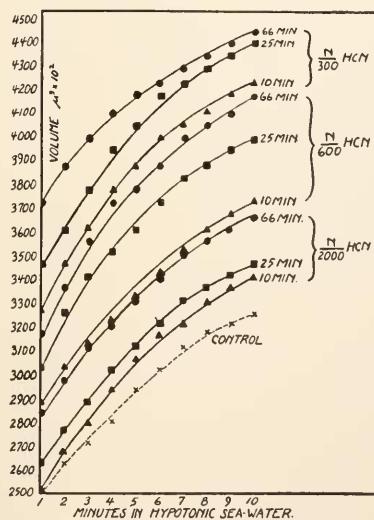


FIG. 2. Illustrating the rate of increase in volume of eggs exposed to the same concentrations of HCN but for varying time intervals. The control was exposed to 100 per cent. sea-water.

rate of increase in volume of eggs exposed to the same concentrations of HCN but for varying lengths of time. Volume here is also plotted against time in hypotonic sea-water. KCN, on the other hand, caused a decrease in the volume of the eggs, when placed in hypotonic sea-water, below that of the control exposed to sea-water. The volume of the eggs varied inversely as the concentrations of KCN. Fig. 3 shows the rate at which the volumes of the eggs decrease with increase in concentration of KCN.

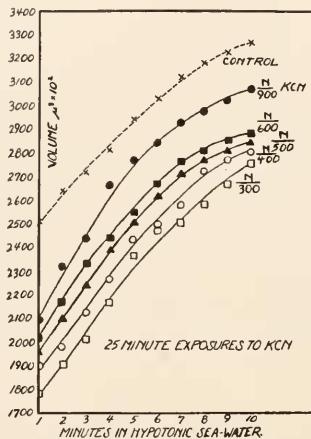


FIG. 3. Volumes of eggs after exposure to KCN showing the rate of decrease in volumes with increase in concentration of KCN. Control was exposed to 100 per cent. sea-water.

The pH of the HCN solutions varied only slightly, being 7.2 to 7.4, while the pH of the KCN solutions remained at 7.4. The work of Lucke and McCutcheon ('26a) shows that the volume of *Arbacia* eggs is independent of the pH of the solution except in cases where the pH is so high or so low as to injure or kill the eggs. According to the same authors ('26b) temperature does have an effect on the volume of the eggs in hypotonic sea-water. Although no attempt was made to keep the temperature constant during the experiments, an accurate record, taken every half hour during the experiments, was kept of the temperature of the laboratory. Conditions at Woods Hole are such that the temperature rarely varied $\pm 2^\circ$ from 22° C.

Eggs which had been exposed to $N/300$ HCN and $N/300$ KCN for 60 minutes, by which time they had reached their maximum

or minimum volumes respectively, were returned to sea-water and fertilized. Cell division took place, showing that the eggs were alive at their greatest expansion or contraction. One cannot very well tell whether an anaesthetized egg can be fertilized while in that condition, since a normal egg does not show first cleavage until about 60 minutes after insemination. Within these 60 minutes, the narcotized condition may have been reversed, and the dividing egg be, not a narcotized egg, but a normal one. The anaesthetized egg, however, did form a fertilization membrane immediately after insemination, and in view of the statement which follows, might indicate that a narcotized egg can be fertilized, but cleavage is delayed until the narcotic has diffused out of the egg, or until the narcotized condition has been reversed. Untreated eggs normally showed first cleavage about 60 minutes after insemination. Eggs which had been anaesthetized with varying concentrations of HCN 3 minutes after insemination and then transferred to sea water, showed first cleavage at varying times always longer than the untreated egg; however, the higher the concentration of HCN used to anaesthetize them, the longer it took for first cell cleavage to appear.

Lillie ('16) has suggested a modification of the equation followed by unimolecular reactions $dx/dt = k(a - x)$, in dealing with rates of osmotic pressure in egg cell, of the form $kt = \ln \frac{V_{eq} - V_0}{V_{eq} - V_t}$ where V_{eq} is volume at equilibrium; V_0 is volume at the first instant (in sea-water); and V_t is the volume at time t . Lillie found that this equation represents the rate of swelling of fertilized and unfertilized *Arbacia* eggs in hypotonic sea-water and Lucke and McCutcheon ('26) found that it applied also to the rate of swelling in sea-water of varying hypotonicity.

That this same equation holds good in the series of experiments described in this paper, can be seen from Fig. 4. When $\log \frac{V_{eq} - V_0}{V_{eq} - V_t}$ is plotted against time in hypotonic sea-water, a straight line should result. This has been found to be the case. The values of k , the velocity constant, are given by the slope of the line. This figure shows that the higher the concentration of

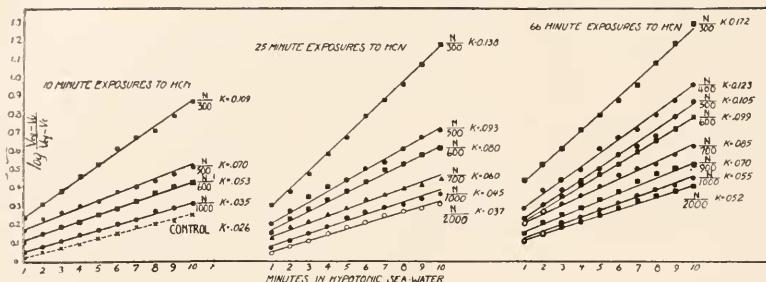


FIG. 4. $\log \frac{V_{eq} - V_t}{V_{eq} - V_0}$ (in which V_{eq} is volume at equilibrium, V_0 is volume at first instant, in sea-water, and V_t is volume at time t), plotted against the time in 50 per cent. sea-water. K , the velocity constant, is obtained from the slope of the lines.

HCN for any given length, the greater is the rate of swelling. Fig. 5 shows the rate at which the velocity constants increase with increase of concentration and increase in time of exposure to the HCN solutions. Fig. 6 is a composite curve in which all the

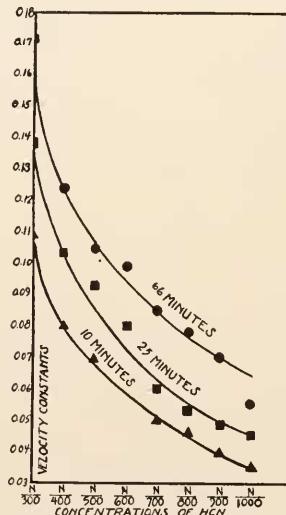


FIG. 5. Showing the rate of decrease of velocity constants with decrease in concentration of HCN.

velocity constants have been plotted to form a single curve. The various velocity constants have been plotted against their respective concentrations. The figure shows that the increase in the rates of reaction proceeds at a regular rate whatever the concentration of HCN and length of exposure.

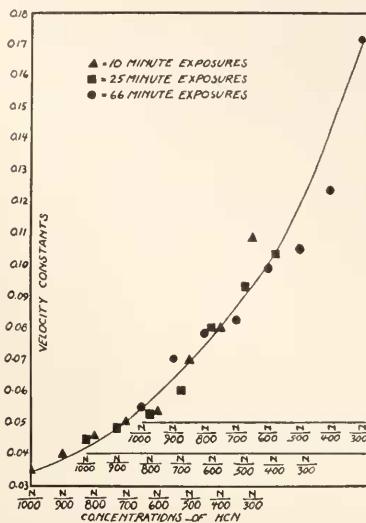
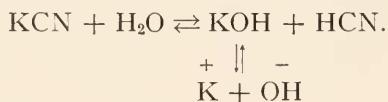


FIG. 6. All the velocity constants plotted against their respective concentrations to show that the velocity constants increase at a regular rate with increase in concentration of HCN and increase in time of exposure to these concentrations.

DISCUSSION.

Lillie's ('13) explanation of the phenomenon of antagonism by various anaesthetics and of anaesthesia in general is based on his experiments which showed that in every case the anaesthetics which he used prevented a general increase in permeability brought about by various salts which are toxic to the cell. That this decrease in permeability by anaesthetics does not always follow, at least when HCN is used as an anaesthetic, is shown by the preceding experiments. Heilbrunn ('25) has also found that ether in various concentrations increases the permeability of *Arbacia* eggs to water. The difference in the results obtained by Lillie and those reported in this paper may possibly be due to the difference in types of anaesthetics used. HCN, as is very well known, dissociates only very slightly. That it enters the cell as a molecule, as does CO_2 [Jacobs ('23)] and H_2S [Beerman ('24)], has been shown by Bodine ('24). Bodine suggests that HCN enters the cell as a molecule and ionizes within the cell to give an intracellular acidity even though the extra-cellular HCN solution is slightly alkaline.

It was shown that HCN is more potent as an anaesthetic than was KCN, since a greater concentration of KCN than HCN is needed to produce anaesthesia. One possible explanation of this follows: KCN in solution is alkaline due to the manner in which it dissociates:



Thus it can be seen that a solution of KCN always contains a certain amount of HCN molecules. Therefore the anaesthetic property of KCN may be due to the HCN molecules present in it, but this HCN is partly antagonized, or its effects interfered with by the KOH formed at the same time. Why HCN should cause an increase and KCN a decrease in permeability is as yet problematic, unless it is the KOH in the KCN solution which is producing the decrease. It is hoped to gather more data on this point in the future.

That HCN acts primarily on the cell membrane and not on the interior of the cell, seems likely in view of the fact that eggs, whether treated with HCN or with sea-water, finally reach the same equilibrium point. From recent work Lucke and McCutcheon (personal communication) state that the velocity of swelling or shrinking of *Arbacia* eggs in hypotonic or hypertonic sea-water is the same, so that Heilbrunn's objection to calling an increase in the volume of the egg an increase in permeability on the ground that it may be an increase in the extensibility of the membrane, does not seem to hold. We would expect, from his statement, that the eggs would shrink faster than they would swell.

SUMMARY AND CONCLUSIONS.

(1) HCN in concentrations varying from *N*/300 to *N*/2,000 causes an increase in the volume of *Arbacia* eggs when placed in 50 per cent. sea-water, the rate of swelling varying directly as the concentration of HCN and the time of exposure to the HCN solutions.

(2) KCN in concentrations varying from *N*/300 to *N*/900

causes a decrease in the volume of the eggs when placed in 50 per cent. sea-water, the rate of decrease varying inversely as the concentration of KCN.

(3) Both HCN and KCN act as anæsthetics, the HCN being more powerful than the KCN.

(4) Anæsthetized eggs can be fertilized while in that condition but it appears that cell division is delayed until the anæsthetic has diffused out of the egg or until the narcotic condition has been reversed.

(5) The formula $kt = L_n \frac{V_{eq} - V_0}{V_{eq} - V_t}$ (where V_{eq} is volume at equilibrium; V_0 is volume at the first instant (in sea-water); and V_t is the volume at time t) correctly represents the rate of reaction.

(6) It is suggested that it is the HCN molecules present in a solution of KCN which causes anæsthesia by that salt, and that its lessened effectiveness is due to the antagonistic (?) action of the KOH which is present, at the same time, in an aqueous solution of KCN.

Appreciation is expressed to Dr. J. H. Bodine for suggesting the problem and for the helpful advice given the writer.

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THE LOW SEX RATIO IN NEGRO BIRTHS AND ITS PROBABLE EXPLANATION.

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It has sometimes been stated that the ratio of male to female births is lower among the Negroes than among whites. The records of births published by the U. S. Census Reports for 1880, 1890, and 1900, gave very low sex ratios for the Negroes,—namely 100.25, 102.67, and 99.80 respectively. The data on births for these years were admittedly incomplete. Births were estimated “by adding to the living children under one year of age as shown by the population returns, the number of those who were born during the year ending May 31, but who died before the end of the year, as shown by the returns of deaths.” The ridiculously low death rates estimated on the basis of the census returns for 1880 and 1890 made it evident that reports of deaths collected during the census year were very incomplete. The deficiencies in the census of 1880 were estimated by Dr. J. S. Billings as about thirty per cent., and those of 1890 were probably even greater. It is quite natural that failures to report deaths would affect most the data on the mortality of infants, especially among the Negroes. That this is the case is evinced by the very small proportion of infant deaths reported as compared with the number of living children under one year of age, whereas it is well known that the actual mortality rate among Negro infants is very high. Inasmuch as male infants suffer an exceptionally high death rate, the effect of basing estimates of births partly on data which leave out a large proportion of infant deaths is to give a sex ratio with too low a proportion of males. On account of the higher infant mortality of the Negroes, to say nothing of more numerous deficiencies in the records, the Negro sex ratio would be reduced more than that of the whites.

Aside from the rather unsatisfactory compilations published in the Census Reports we had, until a few years ago, relatively meager data on Negro births. Dr. J. D. Nichols has compiled records for the District of Columbia (1874-02) and finds a sex ratio in Negro births of 103.10. Beginning in 1915 the annual

reports on birth statistics issued by the Bureau of the Census furnish a sufficiently large amount of data on the sex ratio of Negro births to give very reliable statistical results. The number of Negro births in the U. S. Registration Area from 1915 to 1923 inclusive totals 397,977 males and 386,348 females, giving a ratio of 103.01 males to 100 females. For the same period and area there were born among the whites 5,985,181 males and 5,651,287 females, giving a sex ratio of 105.91. The sex ratio of native-born whites was somewhat higher, 106.072, while that of foreign-born whites was slightly lower, 105.55. The sex ratio of children of mixed marriages, native and foreign-born, showed an intermediate figure,—105.84. The ratios for the Indians and the Japanese were 106.06 and 106.72, respectively, figures very close to the sex ratios of the Caucasians.¹

Does the low sex ratio for Negro births indicate a peculiarity of race? The comprehensive data assembled by Gini point to the conclusion that the sex ratio constitutes a remarkably constant peculiarity of the human species. Before concluding, therefore, that the Negro sex ratio is essentially different from that of the white race other explanations should be sought for. Nichols has made the plausible suggestion that the low sex ratio among Negroes is a consequence of ante-natal mortality. If there is a greater ante-natal mortality among the Negroes than among the whites, and if this mortality is relatively higher in the male sex, there would naturally be a lower sex ratio among the live births in the Negro race. It is a well known fact that the sex ratio of still-births and abortions is unusually high. Data on still-births have been published by the Bureau of the Census for only three years, 1918, 1922, and 1923. These data are naturally very incomplete, and different states have various ways of defining and recording still-births. Nevertheless the data are quite illuminating in relation to the problem in question. The still-births and sex ratios for different groups of the population are shown in the following table:

¹ The data on births for 1924, which have just come to hand, show much the same relations as those quoted. For total live births the ratio for Negroes is 103.98 and for all whites 105.95. The more recent data, therefore, bring the sex ratios for Negroes and whites more closely together. The sex ratios for still-births are as follows: total still-births, 137.48; total white, 137.96; native-born white, 137.81; foreign-born white, 145.26; native and foreign-born, 133.6; total colored, 135.17; Negro, 135.08.

STILL-BIRTHS IN THE U. S. REGISTRATION AREA IN RELATION TO
RACE AND NATIONALITY.

		Total Still- births.	Total White.	Native White.	For- eign White.	Native and For- eign White.	Total Col- ored.	Negro	In- dian.	Jap- anese.
1918	M.	28,127	24,837	15,489	6,413	2,148	3,290	3,257		
	F.	21,507	18,190	11,509	4,466	1,657	2,317	1,482		
1922	M.	39,672	33,593	21,150	8,254	3,190	6,079	5,925	53	91
	F.	29,173	24,739	15,785	5,829	2,374	4,434	4,332	30	59
1923	M.	39,566	33,511	21,232	7,986	3,302	6,055	5,925	37	76
	F.	29,235	24,770	15,840	5,831	2,436	4,465	4,360	32	64
Total	M.	107,365	92,301	57,871	22,653	8,640	15,424	15,107	90	167
	F.	79,915	67,699	43,134	16,126	6,467	11,216	10,174	64	123
Ratio		134.35	137.67	134.16	140.47	133.61	137.52	148.49	140.63	135.78

In all the groups, as may be readily seen, the sex ratio for still-births is remarkably high, and it is especially high for the Negro.

Now for the second point,—the relative proportion of still-births in Negroes and whites. Calculating the ratio of still-births to total births in the two races we find that the ratio is over twice as high among the Negroes as among the whites, namely 7.3 per cent. among the former and 3.5 per cent. among the latter. Syphilis, which is a potent cause of still-births and abortions, is very much more prevalent among the Negroes, and doubtless accounts in no small measure for the high Negro rate of ante-natal mortality.

The facts of differential race and sex mortality in uterine life enable us to explain the low sex ratio of live births among the Negroes without assuming that there is any real racial difference involved. In order to test this interpretation further I have added the still-births and live-births together in the two races and then calculated the sex ratios for total births. As would be expected, the differences between the sex ratios of the two races were reduced. For all whites combined the sex ratio became 106.72, and for the Negroes, 105.54. The inclusion even of the confessedly incomplete data on still-births wipes out most of the difference between the sex ratios of the two races. If complete data on still-births were available, the differences between the sex ratios would probably be reduced to insignificant proportions.

It is perhaps worth while to point out that the sex ratio of offspring resulting from the mating of native-born with foreign-born parents is lower than it is among the native-born. Such matings do not necessarily represent the union of distinct ethnic stocks to a much greater degree than the matings of either the native or the foreign-born, although they probably do so to a certain extent. If we may judge from my studies on the matings falling in this class among the parents of college students,¹ more than fifty per cent. of such mixed marriages would be between persons of the same extraction. On the whole, the federal statistics on births indicate that the sex ratio is little affected by the crossing of different ethnic stocks. There are several factors associated with educational, social, and economic status which probably influence the sex ratio to a greater degree.

The American Negro is to a considerable, but not precisely ascertainable, extent a product of the union of very distinct races. There are no extensive data on the sex ratio of mulatto births as compared with that of the more nearly pure blacks. But since mulattoes are relatively much more numerous in cities than in the country, one may compare the sex ratios of Negroes in urban and rural communities. I have done this for two years, 1922 and 1923, and have added the still-births and live births together. The sex ratio for the cities of the Registration Area is 103.78, and for the rural districts, 106.35. It would be unsafe to conclude, however, that race mixture lowers the sex ratio in this case. The relation is more readily explained by the higher proportion of still-births among the urban Negroes.

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LIVE BIRTHS AND SEX RATIOS AT BIRTH IN THE U. S. REGISTRATION AREA, 1915-22, ACCORDING TO RACE AND NATIONALITY.

	Total Births.	Total White.	Native White.	Foreign White.	Native and Foreign White.	Total Colored	Negro.	Indian.	Japanese
M.....	6,412,118	5,985,181	3,791,433	1,499,368	619,295	426,937	397,977	7,389	18,443
F.....	6,064,740	5,651,287	3,574,396	1,420,482	585,110	413,453	386,348	6,957	17,281
Ratio	105.73	105.91	106.07	105.55	105.84	103.26	103.01	106.06	106.72

THE CHROMOSOMES OF THE INDIAN RUNNER DUCK.

ORILLA STOTLER WERNER.

During recent years the attention of a number of cytologists has been focused on the problem of the avian sex-mechanism. Their investigations have brought varying results and numerous hypotheses have been advanced to explain the cytological findings in such a way as to bring them into agreement with the genetic evidence which is beyond dispute.

Cytologists agree that both physiologically and morphologically the character of the chromosomes of the birds is undetermined and that up to this time our only method of forming a conception of the activities of the chromatic material is through genetic study. In view of this consensus of opinion it would be a hazardous thing for a beginner in the field to criticize the theories that have been developed around the studies of the avian chromosomes unless he has a sufficient number of properly prepared figures for study and has subjected them to a very critical analysis.

Stevens, Hance, and Schiwago agree that the male chick appears to have two large chromosomes equal in size. These they presume are the X-chromosomes. Guyer claims to have evidence to show that in the guinea hen and in the domestic chicken the sperm are of two classes. These are to be distinguished by the presence or absence of an unpaired X-element or accessory chromosome. He believes, however, that the sperm which do not possess the X-element degenerate and are, therefore, non-functional. In regard to this Wilson (1925) says: "In view of this fact it is remarkable that the diploid group seems to show in the female but one large curved chromosome (X) while in the male two such elements are present. The diploid group thus seems to show the expected relations, the female being heterozygous, the male homozygous; but, per contra, the gamete formation of the male seems to show this sex to be cytologically digametic, with one class of gametes non-functional. It is stated,

further, that the X-chromosome of the spermatocyte division is a bivalent body (representing the large pair in the spermatogonia) which passes as such to one pole. If these facts be correctly determined they offer a cytological puzzle with which it is not possible to deal without additional data."

For some time (June 1925 to June 1926) I have been engaged on a study of the chromosomes of the Indian runner duck and I offer the following data as a partial solution of the avian sex-chromosome problem.

MATERIAL AND METHODS.

The material used for giving mitotic figures consisted chiefly of the embryonic membranes of both sexes and to some extent, the germ cells of the male. In most cases the cells of the amnion afforded the best examples, although the cells of the chorion and the allantois gave good results; but such material is more difficult to prepare.

Tissues were obtained from individuals at different stages of incubation ranging from five to twenty-one days. However, those from eight to eleven days were found most suitable for the purpose. Before the eighth day stage it is difficult to determine sex and after the eleventh day the amnion has so completely formed that few dividing cells can be found.

Four general methods of technique were employed; whole mounts from embryonic membranes, stained sections of the embryos, sections of the testes, and smears of the testes.

Membrane Technique.—Of the embryonic membranes the amnion was found to give the best results. This is a very delicate tissue resembling a silk chiffon veil. Because of its delicate structure it was found best to keep it as nearly intact as possible. In this way the pressure of the amniotic fluid kept it stretched while being fixed and thus most of the tissue could be saved. Of the allantois only parts could be used. This was especially true of embryos of longer incubation, for the heavy blood vessels catch and hold the stain and the tissue is thick and becomes hard.

Great care was taken that the eggs should not become chilled while being removed from the incubator. The embryo within its amnion, and sometimes a part of the allantois, was removed from

the egg with warm instruments and placed in Allen's modification of Bouin's fluid for two hours. The temperature of the fixative was kept at 37°. The amnion was punctured at the end of an hour to allow the fixative free access to the embryo.

The tissues of the first three embryos were rendered practically useless by increasing the strength of the alcohol too rapidly. The chromosomes were clumped and massed so that it was almost impossible to make a count. For this reason the following procedure was worked out. When the tissues were removed from the fixative they were rinsed in several changes of distilled water at a temperature of 37°, then passed successively through the following grades of alcohol: $\frac{1}{2}$, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 per cent. During this time the alcohols were maintained at the same temperature and the tissues were allowed to remain approximately twenty minutes in each fluid. While the material was in the 10 per cent. alcohol the membranes were removed from the embryos, and placed in a 2 per cent. solution of iron alum; one and one half hours. They were then rinsed in several changes of distilled water and placed in an aqueous solution of Heidenhain's hematoxylin, two hours; rinsed in tap water and destained in iron alum. They were then passed successively through the following grades of alcohol, remaining about ten minutes in each grade: 12, 14, 16, 18, 20, 22, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90 and 100 per cent. They were then passed through xylol, fifteen minutes, cedar oil three hours, back to xylol ten minutes, 100 per cent. alcohol one hour, forward to cedar oil two hours, then xylol fifteen minutes, and finally cut into small pieces for mounting. The double clearing makes the tissues beautifully transparent.

As suggested by Painter in his study of mammalian material, the large mesodermal cells were found to be the best for study. For this reason the tissue was placed on the slide with the mesodermal surface upward. They were mounted in gum damar, a small leaden weight being placed on the cover slip while the slides were drying.

Technique for Embryos.—The embryos were taken from the ten per cent. alcohol and passed through the same grades as were the membranes but because of their greater bulk were left thirty minutes in each grade. They were then passed through half

100 per cent. alcohol plus half xylol, 15 minutes; pure xylol, five minutes; one half xylol and one half 48° paraffin, fifteen minutes; then embedded in the usual way. They were cut at seven micra and stained with Heidenhain's hematoxylin and the work completed as is usual for such material. These slides were used in determining the sex of embryos whose membranes had furnished cells for study.

Technique for the Sectioned Material of the Testes.—The testes were from eight-months-old individuals. On removal from the body they were cut into millimeter cubes and dropped into the same fixative as above as soon as possible. They remained in the fixative two hours at 37° then were washed in several changes of distilled water at the same temperature. The material was now passed through the following grades of alcohol, two minutes each: $\frac{1}{2}$, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 18, 20, 22, 25, 30, 35 and 40 per cent. At this stage all the alcohol was drained off except enough to cover the tissues. Then by means of a pipette, equal parts of bergamot oil and 50 per cent. alcohol were dropped in. The mixture was stirred constantly by bubbling it with a pipette. Again all the liquid was drained off except just enough to cover the tissues, and in each of the following changes this rule was observed. Equal parts of 60 per cent. alcohol and bergamot oil; equal parts of 70 per cent. alcohol and bergamot oil; pure bergamot oil. The old oil was now drained off and fresh bergamot oil dropped in. This process was repeated several times until all the alcohol was removed. The tissues were then left in the pure bergamot oil two hours (the tissues may be left in this oil for several hours without injury). The oil was then drained off and three fourths parts of bergamot oil and one fourth part of oil of wintergreen were dropped in, then one half bergamot and one half wintergreen, one fourth bergamot and three fourths wintergreen, pure wintergreen. Then the old wintergreen was drained off and fresh added several times until no bergamot remained.

From the oil of wintergreen the tissues were carried by steps through to paraffin. Sixteen bottles were used, starting with one part paraffin and fifteen parts wintergreen and finally ending in pure paraffin. Then the tissues were passed through several

changes of pure paraffin to remove all the wintergreen. They were then placed in an oven for twelve hours, then in fresh pure paraffin for one half hour and finally embedded. While the tissues were being passed through the paraffin, the temperature was kept just so the paraffins would stay liquid, care being taken not to raise it above this point. Sections were then cut about five and one half micra. I think, however, that it would be better to cut them thicker than this, say about seven or eight micra. In this way many more cells could be found with none of the chromosomes sectioned away.

After the tissues were mounted the work was completed according to the usual methods.

The chromosomes in these cells stand out clear and beautiful.

The destaining is a delicate process and must be done with extreme care. No definite time can be given for the iron alum in destaining for the time depends upon the thickness of the sections, the concentration of stain used, etc. Experience alone brings the desired results. The work is best done under the low power of the microscope (10 \times ocular and 2/3 objective).

Technique for Testis Smears.—These were prepared just as the sections but the oils and the paraffins were omitted. From the 40 per cent. alcohol they were passed successively down through the grades of alcohol, care being taken to gradually lower the temperature of the fluids until when the work is completed they are at room temperature. This can best be accomplished by placing all the containers on an electric embedding plate.

The study of the avian chromosomes has presented difficulties in that the chromosomes are easily massed together, making a count difficult or impossible. With the technique here employed this difficulty has to a considerable degree been avoided.

Approximately six hundred cells were examined. The greater portion of these were soma cells from the tissues of nine individuals. In determining the number of chromosomes I have, in most cases, made drawings from several different slides. However, where the cells were found particularly clear and distinct I have made several drawings from the same slide.

The chromosomes were measured in the following manner. A separate camera lucida drawing was made of each. A thread

was moistened and laid along the median curvature of the chromosome outline. By means of a razor blade its ends were cut at points corresponding to the boundaries of the ends of the chromosome as the thread lay upon the drawing. The segment was then removed and drawn out straight and measure taken in millimeters. In this way with a considerable degree of accuracy the length of the chromosomes was obtained.

CHROMOSOMES OF THE SOMATIC CELLS.

In the duck it will be more convenient to consider first the complex of the male (Figs. 1 to 7 and 43 to 48) since it is in this sex that the number of chromosomes is even.

There are probably seventy six. Some deviation from this number was found in the early part of the work. This was due, in greater part, to a failure to recognize the small globe-shaped chromosomes. For a time they were thought to be basophylic knots on the filaments that sometimes occur between the smaller chromosomes. As the work progressed, however, it became more apparent that these were true chromosomes. In addition to this deviation about half a dozen cells were found in the amnion in metaphase that appeared to have but half the usual number of chromosomes (thirty-eight). At this time no explanation can be offered for this condition. With the exception of the above few cases all cells examined, in which the chromosomes were well separated, seemed to have seventy-six. I am inclined to think that this is the basic number.

These seventy-six chromosomes fall readily into three general groups: six pairs of large chromosomes, including three J-shaped and three rod-shaped; nine pairs of short rod-shaped chromosomes; and twenty-three pairs of globe-shape. This may be seen in Figs. 43-48, Plate 8, which is an artificial pairing of these chromosomes arranged according to size. The morphology and the length were taken as a basis for this pairing. Where there is a slight difference in the lengths of the members of a pair, the shorter member is usually broader at one end than its mate. This makes the chromatic material of each member approximately the same.

Of the six pairs of large chromosomes (Nos. 38-33) the largest

are J-shaped bodies (Figs. 3, 4, 6, 7 number 38). Next in size are a pair that are usually of rod-shape (Nos. 37) only slightly shorter than the longest in the complex. Pairs 36, 35, 34, and 33 form a graduated series in which there is but little difference in the lengths of the consecutive pairs (Figs. 1-7 and 43-48). However, there is a greater difference between pairs 34 and 33 than between the other pairs. Pairs 36 and 35 are of the J-type (Figs. 5, 6, and 7), and 33 and 34 are usually of rod-shape or bent rods, though in some prophase cells they seem to be J's (Figs. 3, 7, 44 and 45).

The J-chromosomes (Nos. 38, 36, 35) of this group are, in most cases, constant in shape throughout prophase and metaphase and, as far as observed, in anaphase. The three pairs of rod-shaped chromosomes (37, 34, 33) assume various forms according to the stage of mitosis and according to their position in the nucleus. The members of the largest pair (Nos. 37) are especially interesting in this respect. In the metaphase they are, in most cases, straight rods pointed at the proximal end (Fig. 3). At times, however, they appear in the form of U's (Fig. 4). If they lie near the nuclear wall they bend to accommodate themselves to this limitation. One or both ends may be bent (Fig. 7). Also these two bodies are not always bent in the same shape in the same cell but are so nearly the same in size that one may recognize them among the other chromosomes with a fair degree of certainty. Fig. 23 shows some of the shapes that they assume. There seems to be some differentiation of the chromatic material near the ends of these chromosomes which gives them increased flexibility at these points. This differentiation seems to be a suture accompanied by a slight constriction of the chromosomal wall. One of these sutures, if it is such, is near the incurved end when the chromosome lies as represented at *a*, *b*, *c*, *d*, in Fig. 23. The other point of differentiation is at the opposite end of the chromosome and marks off a portion of the chromosome about three times the length of the former end part.

With reference to the matter of sutures and constrictions, the condition found is not peculiar to the duck for such has been described by many observers in both animals and plants. These sutures and constrictions, it seems, may be median or at any

other point and in some cases at least, they are constant in position. For example, Sakamura ('16) found that in *Vicia* several of the chromosomes show a constant subterminal constriction and that those of one pair show a constant median constriction in addition. However, Agar ('12) found in *Lepidosiren* that these sutures vary in position in different chromosomes and that they correspond with the points of attachment to the spindle and that their position in the chromosome of the meiotic division corresponds with that in the spermatogonial chromosomes. Wilson, however, points to the fact that these sutures and constrictions are visible in the prophase before the spindle is formed and therefore are not caused by the attachment. That these sutures may not be necessarily connected with the attachment of the traction fibers is shown by the No. 37 chromosomes of my material. Here there are two sutures, one at about one twelfth of the length of the chromosome distant from one end and the other at about one fourth the length of the chromosome distant from the other end. It is hardly probable that there would be two traction fibers so widely separated arising from the same somatic chromosome.

Again it should be pointed out that the permanence of position of such sutures at at least two different points along such chromosomes (see Fig. 23) would seem to be morphological evidence that such chromosomes differ in a qualitative way throughout their length.

So far, we have been following the characteristics, sizes, etc., of the largest chromosomes. Let us next consider the characteristics of the chromosomes of the intermediate group. There are nine of these (Nos. 32 to 24) and they are of the short rod-shape type. Among these is one pair, the members of which are sometimes seen as crescent-shaped bodies (Fig. 8, cr.). Altogether the nine pairs form a closely graduated series ranging in size from those somewhat shorter than the shortest in the foregoing series to short ones that are almost as thick as they are long, but certainly distinguishable as rods by their acute angles (Figs. 43-48).

The third group of chromosomes consists of forty-six round or globe-shaped bodies (numbers 23-1). These also form a closely

graduated series. Only twenty-two of these are of sufficient size to permit their being paired. The remaining twenty-four are small and so closely graduated in size that it is impossible to pair them with any degree of certainty.

The nine pairs of short rods and the twenty-three pairs of globe-shaped chromosomes are remarkably constant in shape during the progress of prophase from the time they are discernible as individual bodies until they enter the equatorial plate in metaphase. Here they mass together to such an extent that frequently they are no longer distinguishable as individual bodies. But even in this phase it frequently happens that they may be seen in their characteristic forms.

There appear to be seventy-seven chromosomes in the cells of the female (Figs. 8-22, 37-42). The longest of these is a large unpaired body, larger than any of the other chromosomes, and on account of its size, in most cases, easily distinguishable from the other chromosomes (Fig. 37-42, W). In cross section it is large at one end and taper at the other. In the earlier stages of mitosis it is a more or less bent rod, seeming to accommodate itself to the other nearby chromosomal regions and also to the nuclear wall (Figs. 8, 10, W). In the late prophase and the early metaphase it continues to be a more or less bent rod (Figs. 9, 11). In Fig. 17 it is a rod bent upon itself. Figs. 14 and 22 show it as U-shape while 16, 19 and 20 show it with bendings in different regions. In Fig. 12 it is a rod somewhat foreshortened. It would seem that this chromosome possesses a great degree of flexibility throughout its length which permits its bending in various ways.

The next largest chromosomes in the cells of the female take at times the form of curved rods but more frequently are of J-shape (Figs. 8-22, 37-42; 38w, 38Z). Whether they are gonal mates or not it is difficult to say. Theoretically, as will appear later, they should not be. From their size and their J-shape one would suppose that at least one of them is a homologue of the 38's in the cells of the male. The other might be of the nature of a w-chromosome.

Slightly shorter than these are two large curved rods (Nos. 37) one of which in prophase and early metaphase quite frequently takes the form of an S (Figs. 9, 17). The members of this pair

resemble the pair number 37 in the cells of the male in sutures and constrictions (Fig. 23 a-d), and like them they assume various shapes (Fig. 23, e-i).

The remaining seventy-two chromosomes in the cells of the female (Nos. 36-1, Figs. 37-42) are approximately the same with respect to behavior, size relations, etc., as those in the cells of the male (Figs. 42-48).

In my study of the somatic cells I have been increasingly impressed with the evident pliancy of the chromosomes. They appear to bend to accommodate themselves to the nuclear wall and to other chromosomal regions. In most of the chromosomes (with certain exceptions to be mentioned later) the degree of pliancy seems to be about equal in all their parts. For this reason the morphology of chromosomes of the same length which are probably synaptic mates is not always the same. The ultimate conclusion regarding the shape of each particular chromosome, I believe, must be drawn from its appearance in metaphase where it is entering the equatorial plate and even in this phase some may be atypical in shape because of the obstructions of other chromosomes. The atypical forms, of course, have been found a lesser number of times and the conclusions are based on the larger number of cases.

The most convincing evidence is found in a comparative study of the chromosomes when those from different cells are arranged in serial order and in tables as in Plates 7, 8, and 9. Figs. 37 to 42 show the chromosomes from cells of the amnion of females; Figs. 43 to 48 from the cells of the amnion of males; Figs. 49 to 54, Plate 9, show first-spermatocyte chromosomes from smear preparations of adult testes. In these tables the constancy of the size and shape of the individual chromosomes stand out at once. There is some variation in form of the chromosomes from cell to cell but it is due, I believe, as previously stated, to their pliancy and in some instances to the particular angle at which the chromosome lies relative to the observer, and probably more than anything else to the particular stage of mitosis in which the cell happens to be found. Figs. 37, 38 and 40 are taken from early prophases (Figs. 8, 9, 10); Figs. 39, 41 and 42 are from prophases of later stage (Figs. 13, 12, 16); Fig. 43 is from a cell in early

metaphase (Fig. 4); Fig. 47, and 48 are from late prophanes (Figs. 1, 2).

The morphological groups and the size relations within the groups are very evident in this comparative series. The distinction in size, etc., between the group of twelve (thirteen in the female) large chromosomes (38-33) and the group of 18 of intermediate size (32-24) is clearly marked in Plates 7 and 8. This is especially true in Figs. 37, 38, 40, 43, 44 and 45. It may also be plainly seen that the intermediate-sized and the globe-shaped chromosomes each form a finely graduated series. Because of this finely graduated condition and because there are so many of them there may have been some inaccuracy in the pairing and, in the case of the globe-shaped series, possibly in the count also, for these, the smallest chromosomes, frequently overlie one another. But it is certain that there is no inaccuracy in the count in the cases of the members of the larger and of the intermediate-sized groups. There is clearly 12 plus 18, or 30, of these bodies in the cells of the male and 13 plus 18, or 31, in the cells of the female. Neither can there be any doubt as to the presence of at least one unpaired chromosome among those from the female, for it is larger than and morphologically different from the other chromosomes. In the case of the chromosomes which have been arbitrarily numbered 38w and 38Z it might be said one is dealing with gonial mates, since they are similar in length and contain approximately the same amount of chromatin. These facts, however, considered alone, are not sufficient proof that they should be considered homologous rather than that they might be, as I shall attempt to prove later, two additional odd chromosomes which are concerned with the sex-mechanism. The important point in this paper is the fact, previously indicated, that a study of the cells of the male reveals an even number of the larger chromosomes while a study of the cells of the female shows an odd number and one more than in the cells of the male.

The morphological difference of the last group of chromosomes (globe-shaped) from the two preceding groups is not a condition peculiar to the duck. Painter shows the same condition in the lizard (Figs. 35, 36). As previously stated the twenty-two smallest chromosomes are so small and so closely graduated as to

size that it is impossible to pair them. I have placed them in these plates simply to finish the series. In the first spermatocyte cells (Figs. 24-29) these globe-shaped chromosomes are picked out with less difficulty because they are fewer in number; but here the tetrad condition often interferes with distinguishing size relations. However, where a large number of such cells are studied a considerable degree of certainty concerning numbers, morphology, and size relations may be obtained.

CHROMOSOMES OF THE SPERMATOCYTES.

The preliminary process of reduction is initiated by syndesis in the course of which the chromosomes become closely associated two by two to form bivalents or gemini. This is, of course, only a pseudo-reduction producing a haploid group of bivalents each representing a pair of chromosomes. Because of this fact we would expect to find in the first spermatocytes where such phenomena occur one half the somatic number of chromosomes.

In counting, the prophase stages were found to be most suitable. Here the chromosomes lie well apart, which facilitates, not only the counting, but also a study of the forms of each, especially of the large tetrads (Figs. 24-29). Smear preparations were most suitable because whole cells could be used with the resulting certainty that all the chromosomes were present. Metaphase figures were also studied but no drawings of these are here used on account of the tendency of the small tetrads to crowd together in the center of the plate. The large chromosomes, however, stand out clearly in metaphase and have a tendency to occupy the outside of the circle as in the somatic cells (Figs. 1-22) and as in similar cells of other species (Figs. 35, 36).

Figure 24 represents a particularly clear prophase. In Fig. 49 the chromosomes of this cell are artificially arranged according to their size. In these bodies the tetrad form can be made out in most of the larger members, and at least the bivalent condition may be seen in the smaller members of the series. This is also true of Figs. 50 to 54. Attention is called to the similarity of the size relations among the members of the spermatocyte series to that of the homologous pairs in the somatic series (Plates 7, 8).

There is a distinct drop in size between the sixth and the seventh largest tetrads (Nos. 33 and 32). The same size distinction may be noted between numbers 33 and 32 of the soma cells. There is a similar distinction between the 35's and the 34's in the spermatocyte and somatic series. The size relations among the shorter spermatocyte rods (Nos. 32-24) and the bivalents derived from the globe-shaped chromosomes are apparently about the same as in the soma cells. In each case they form a similarly graduated series.

In addition to the resemblance in size between the spermatocytic and the somatic series there is also a striking similarity in form. Tetrad number 38, the largest in the group (Fig. 24), shows that it has arisen from a pair of J's similar to those seen at 38Z in Fig. 43, Plate 8. In Fig. 25 the long arms of the J have not yet disjoined; the shorter arms have. This chromosome is well shown in Fig. 52 where the disjoining is almost complete. Chromosomes 36 and 35 also show that they have arisen from a pair of J's (Fig. 25). In each of these chromosomes the long arms of the J's have not disjoined while the shorter ones have. Chromosome number 37 (Figs. 24, 25, 26, 27, also Plate 9) shows constrictions similar to those found in number 37 in the soma cells, although these are more apparent in the tetrads, *i.e.*, the constrictions are deeper. In some of these cases only one of the constrictions is apparent but whether one or both are present, when they do appear, each seems to be in about the same position in the members making up the tetrad that it would occupy in the somatic homologues.

GONOMERIC GROUPING.

The probable existence of an odd chromosome in one of the gametes, as well as a definite number of the large chromosomes in each of the gametes, is further evinced in somatic cells in the grouping of the chromosomes in early metaphase. I have examined numerous cells in metaphase in both sexes. In the cells of the male the large chromosomes are grouped six on one side of the forming equatorial plate and six on the other (Figs. 3, 5, 6). In the cells of the female there are six on one side of the plate and seven on the other (Figs. 17, 18, 19, 20, 21, 22). In every case

there is in the group of seven, one chromosome which is larger than the others which has the characteristic form of the largest odd chromosome in the cells of the female, large at one end and taper at the other.

Schiwago (1924) has recognized gonomeric grouping in the cells of the domestic chicken. (I have taken the liberty of reproducing some of his drawings as well as those of Hance from the same form and two from Painter from the lizard). The lettering of the chromosomes in Figs. 32 and 33 are as Schiwago gave it. The same grouping is apparent in the work of Hance and Painter although the authors do not point out the fact. I have again taken the liberty to draw a line through each of these figures (Figs. 30, 31, 35, 36) which might suggest a possible separation of the chromosomes into maternal and paternal groups.

D. H. Tennent (1908) found gonomeric grouping when working on the eggs of *Toxopneustes* and *Arbacia* fertilized with *Moina* sperm. Margart Morris (1914) in her work on hybrids between *Fundulus* and *Ctenolabrus* found a somewhat similar condition. Many other instances of this phenomenon among different species are on record.

Gonomery represents no more than a tendency on the part of the chromosomes to remain in separate maternally and paternally derived groups during a part of the early development. Considering these facts, such a phenomenon in the duck would seem to afford a valuable check on the count of the chromosomes, as well as a check on the number contributed by each sex, especially with respect to the large chromosomes, since these are so well defined that they may be readily recognized.

FILAMENTOUS LINKAGE.

Filaments which seemed basophylic in character were found connecting chromosomes in somatic cells in prophase stages. Not all groups of chromosomes formed by such fastenings were constant. Some groups composed of definite numbers were found a sufficient number of times to justify one in concluding that they might be relatively constant. In nearly all cases the groups are made up of chromosomes of the intermediate and smaller

sizes. In some cases chromosomes especially of the short rod-type might be connected by two such filaments in such a way as to extend one from either corner of the end of an individual chromosome and attaching in like manner to the end of the next chromosome. In other cases both filaments might be fastened at the same point on a rod-shaped or a globe-shaped chromosome (Fig. 10).

Of the inconstant groups one composed of eighteen small chromosomes may be seen in Fig. 9. In the upper part of this figure there is a group of two chromosomes attached to one of the large **J**-shaped chromosomes. Fig. 13 shows a group of 13. Sometimes filamentous fastenings occur between the smaller chromosomes when these bodies lie in what is, apparently, a linear arrangement. However no cells have been observed in which all the chromosomes so arranged were fastened by filaments. If such a condition exists, it would be exceedingly difficult to discover since the chromosomes so arranged lie close together and are usually at two foci, the one lying over the other or nearly so.

Of the groups having the more constant types of fastenings one may be seen in Figs. 1, 9, 10 and 12. In this group ten of the chromosomes belonging to the intermediate series, seem to be connected end to end by interchromosomal filaments in such a way as to form two chains of five chromosomes each, consisting each of two rods and three globes. The members of the one series are similar in size to those of the other group. There are also in late prophase, four chromosomes belonging to the intermediate series connected end to end in such a way as to form two chains of two chromosomes each (Figs. 1, 9, 12).

Quite often the greater number of the small chromosomes appear to be in linear arrangement, I believe that in some cases they extend in a circle within the nucleus. In late prophase there seems to be a row extending almost across the space within the circle of the large chromosomes (Figs. 8, 9, 12). In some cases this row bends a little as a line appears when it extends around a globe. Manipulation of the fine adjustment shows that another row similar to this one lies at a deeper focus (Fig. 12) apparently at the opposite side of the nucleus. (It must be borne in mind

that I was examining entire cells, not sections of cells.) These rows may extend in any direction across the nucleus, but they always lie within the circle of the larger chromosomes.

DISCUSSION.

The chromosome number in the somatic cells and early germ cells is traceable half of it to the paternal parent and half of it to the maternal parent. There is, then, in the typical cell, an even number of chromosomes. If in any form an odd number is found, the question is immediately presented as to the disposition of the odd chromosome in maturation and what part it might play in the transmission of hereditary factors.

An attempt to solve the problem of the odd chromosome in the case of the Indian runner duck, as well as in any other form, necessitates not only a tracing of its disposition or probable disposition in maturation and zygote formation but also a discovery of a parallelism with the vast amount of results obtained from experimental breeding.

In the Indian runner duck the male has an even number, seventy-six chromosomes, but the female has an odd number, seventy-seven, and strange to say, one more rather than one less than is present in the male. Of course where the odd number occurs in the female, one would immediately look to the chromosomes in polar body divisions for an explanation. The difficulty of getting eggs in the right stage of maturation is at once apparent. It seems best, therefore, at this time to attempt an explanation of the phenomenon from indirect evidence and in the light of the results of the work on Lepidoptera, in which the female is known to be digametic for sex as is probably the case in the aves.

In the duck the additional unpaired chromosome present in the cells of the female is without a homologue in the cells of the male and of course could not then be a sex-linkage chromosome. If it is not concerned with sex-linkage it must probably belong to the class of sex-determining bodies known as the W-chromosomes, occurring only in females.

With this chromosome (W) ruled out of consideration it then becomes the problem to find which one of the remaining chromo-

somes in the female is concerned with sex-linkage and finally to account for the presence of a third non-homologous body which must evidently be present.

Sex-chromosomes carrying sex-linked characters are usually among the largest of the complex. In the male of the Indian runner duck, then, it is reasonable to suppose that the sex pair would be among the largest if not the largest in the series. In this series there are certainly six pairs that are sharply marked off in size from the remaining thirty-one pairs. Among them again three pairs may be distinguished as larger than the remaining three, and the largest of these is a pair of J's which it might be supposed are the sex pair. For the sake of argument, at least, let us assume that 38Z is the sex pair (Figs. 37-42, number 38Z).

Now, in the female the sex-chromosome concerned with sex-linkage should be an unpaired body and similar in size and shape to the sex pair in the male with the members of which it, of course, would have to alternate. Evidently the largest chromosome (W) can not be this body. If the sex-linked chromosomes in the male are the largest in the series, as is usual in animals, in the cells of the female we would of necessity look for a homologue of them among the largest in the series after the longest chromosome (W) is ruled out. From the drawings it would seem that there are four of these next largest chromosomes and they are very much alike in size. Just what one of the four might function in the capacity of a sex-linkage body is not possible to determine now. It might be supposed, however, that, as in the male, it is one of the largest (38Z).

If that be the case there would then remain an unpaired chromosome in the female to be taken from the remaining three large chromosomes. This chromosome like the largest one (W) in the female series would have no homologue in the male series and of course would be limited to the female line and would be a second w-chromosome (w).

In the female complex, then, there would be a group of three large chromosomes, one of which would be a sex-linked chromosome, homologous to and capable of alternating with the largest sex-linked chromosomes in the male. The other two would be chromosomes not concerned with sex-linkage and, since they

remain in the female determining gametes, would be concerned only with sex-determination.

If the large unpaired chromosome in the cells of the female is one of a complex of three that go to make up the sex-determining mechanism, the condition in the duck might then be a case similar to that in *Phragmatobia*, one of the Lepidoptera, which was described by Seiler (1914).

In *Phragmatobia* during polar body formation a single element was seen to separate from a large heteromorphic element that consisted of two unequal chromosomes yoked together. After separation the heteromorphic element fragmented into two unequal chromosomes at one pole. As the result, half of the eggs got the large single element and half got the two unequal chromosomes.

If the duck be like *Phragmatobia*, the unpaired chromosome here designated as Z might be thought comparable to the large element in *Phragmatobia* and some two of the other large chromosomes, here designated W, w, might be comparable to the heteromorphic element in *Phragmatobia* which was seen to be distributed to one pole in maturation and to break into two chromosomes.

SEX-DETERMINATION AND SEX-LINKAGE.

Wilson (1925) is of the opinion that "all the difficulties" in regard to an explanation of the function of sex-chromosomes in sex-determination "disappear" if we assume that in any particular species there is but one kind of X-chromosome, in itself neither male-determining nor female-determining, but so adjusted to the general mechanism of development that when single it swings development toward the male side, when double toward the female side. This view essentially quantitative ascribes to the egg the capacity to produce either the female or the male, according to the presence of more or less of the X substance."

On the other hand, Bridges (1922) from his study of triploids in *Drosophila* concludes that "both sexes are due to the simultaneous action of two opposed sets of genes, one set tending to produce the characters we call female and the other to produce the characters called male." According to his hypothesis there is a preponderance of male tendency genes located in the autosomes

and a preponderance of female tendency genes located in the X-chromosomes. In the presence of one X the male-tendency genes located in the autosomes overbalance the female tendency genes in the single X-chromosome and a male results; on the contrary the presence of two X-chromosomes overbalances the male-tendency genes in the autosomes and the development is thrown toward femaleness.

Goldschmidt (1923) in his work on moth hybrids has come to the conclusion that the female producing factors lie in the W-chromosome. In crosses between certain races females are gotten through non-disjunction in such a way that the W-chromosome comes from the father and the Z-chromosome comes from the mother, just the reverse of the usual method. In such cases he finds that femaleness follows the W-chromosome.

In the light of the results obtained by Bridges on sex-intergrades in *Drosophila* produced through triploidy or otherwise unbalanced conditions of chromosomes, it is very clear that sex is not controlled entirely by a particular chromosome either singly or doubly represented as Wilson would say. There may be genes in many of the autosomes as well as in the sex-chromosomes which individually tend to throw the balance toward maleness or toward femaleness. But Bridges was dealing with an X-Y type of sex-determination and with male digamety. His opposing tendencies are located in the X-chromosomes and the autosomes. In addition to this his X-chromosomes carry sex-linked characters. Bridge's scheme as may be seen, will not work for the birds or moths where female digamety occurs. But if we adopt the suggestion of Goldschmidt (1923) that the W-chromosome which passes from mother to daughter carries, female-tendency genes only and in addition assume that male-tendency genes are located in the Z-chromosomes of which two occur in the male and one in the female, we have a mechanism that in many cases would seem to take care of both sex-linked characters and sex-determination.

In the duck then, if the large unpaired element in the cells of the female is one of a complex of three that go to make up the sex-determining mechanism, the following scheme would be entirely in accord with the parallelism of cytology and experimental breeding.

In the male there are seventy-six chromosomes. It would seem then that seventy-four of these are autosomes and two are Z-chromosomes. In maturation each sperm would get thirty-seven autosomes, plus one Z-chromosome, or thirty-eight in all, and all male gametes would therefore be alike.

In the female there are seventy-seven chromosomes. Comparing these with the conditions in the male it would seem that there are seventy-four autosomes, plus one Z, like those in the male, plus two W-chromosomes which are not present in the male.

In maturation it might be supposed that the two W's become linked together and that one of these might pair with the Z-chromosome, the whole resulting in a tripartite body. If the thirty-seven pairs of autosomes behave as in the male there would result gametes in the female of two sorts (Figs. 55, 56) one having thirty-seven autosomes plus the Z and the other having thirty-seven autosomes plus the Ww pair. At the formation of the zygotes then, those eggs having thirty-seven autosomes plus the Z, fertilized by a sperm having thirty-seven autosomes plus a Z would result in a zygote having seventy-four autosomes plus two Z's or a male. Those eggs having thirty-seven autosomes and two W's fertilized by a sperm as before having thirty-seven autosomes plus a Z would result in a zygote having seventy-four autosomes plus one Z and two W's or seventy-seven chromosomes and would be a female.

In sex-linkage the Z-chromosomes alone would be concerned. If the double lined Z, represented in Fig. 55 as occurring in the male, is thought of as carrying a dominant sex-linked character and the single lined Z here represented as occurring in the female, be thought of as carrying a recessive sex-linked character, then in the F_1 generation, both males and females would show the dominant sex-linked characters and would be like the male of the parental generation. But in the F_1 generation all males would be heterozygous for the sex-linked characters and would form gametes of two sorts, half carrying the dominant sex-linked character and half the recessive.

In the F_2 generation all males would show the dominant sex-linked character, but of the females of this generation, half



would show the dominant sex-linked character and half would show the recessive character, the female inheriting it in each case from the male parent of the F_1 generation.

According to this scheme it might be supposed that in the case of the Indian runner duck each autosome sustains a balanced condition of the male and female-tendency genes. The Z-chromosomes might be thought of as carrying a preponderance of male-tendency genes. An individual, then, receiving two Z's would become a male. The two W's might be thought of as carrying female tendency genes only. These, however, would be thought of as being quantitatively greater than the male tendency genes carried in a single Z and an individual receiving a Ww plus one Z would become a female.

A large number of animals belong to the X-Y type in sex-determination. In these the female has an even number of chromosomes, the X-chromosome being paired or diploid. The male has one less than the even number of chromosomes found in the female, the X being unpaired. This gives the female more chromatin than the male. In a few of the animals of the WZ mechanism, such as some of the moths, the male has an even number of chromosomes. The female has the odd number and one less than the male which would give the male more chromatin than the female. But in most of the cases in the moths the female has one more chromosome than the male, the male having the even number.

In the birds, if my observations are borne out on other species, it would seem that the female has the preponderance of chromatin. If the conditions in the duck are representative of that which one might expect to find generally in the aves, we would expect in the moths which are likewise digametic in the female, that the true state of affairs would be like those moths in which the female has one more chromosome than the male. The difference between the two types of animals, the XX-XY and the WZ-ZZ, is essentially in the matter of which sex produces the two sorts of gametes. And so far as this mechanism is concerned it is a matter of indifference which sex produces the two sorts of gametes. The important thing seems to be that one sex, usually the female, should have more chromatin than the other.

SUMMARY.

1. This study was undertaken with two points in view: First, to determine the number of chromosomes and second, to discover the mechanism for sex-determination and sex-linkage in the duck as a representative of the aves.
2. Material from thirteen individuals was used, seven males and six females.
3. Chromosome counts were made on entire cells, both somatic and spermatocytic.
4. In the somatic cells there appear to be seventy-six chromosomes for the male and seventy-seven chromosomes for the female. There is present in the cells of the female a long unpaired chromosome which is not found in the cells of the male. There is reason to suppose that there are probably among the remaining six largest chromosomes two more unpaired chromosomes, one of which, the largest, is probably homologous to the largest pair (sex-linkage) of chromosomes in the male complex, while the other, it is thought may be some one of the five remaining long chromosomes.
5. There appear to be 38 bivalents in the primary spermatocytes of the male. These agree with the pairs of somatic chromosomes in size gradations. Most of the chromosomes in the spermatocytes appear to be bivalent or tetrad in form.
6. Sutures accompanied by constrictions seem constant in position in at least two points in the largest rod-shaped chromosomes. These sutures seem to lend additional pliancy to these regions in the chromosomes.
7. Gonomeric grouping of chromosomes occurs in the amnion cells of the duck. It is also thought to be present in the chromosome plates of the gonial cells in the embryo of the chick and the lizard.
8. Filamentous linkage between certain of the smaller chromosomes appears to be present in certain stages of the prophase.
9. There is reason to believe that the sex-mechanism is of the $WwZ-ZZ$ type similar to that found in the moth *Phragmatobia*.
10. This would give the female more chromatin than the male and yet preserve female digamety, which would bring this type into harmony with the usual conditions found in the $XX-Xy$ type.

Acknowledgment is hereby made to Dr. W. R. B. Robertson, at whose suggestion this problem was undertaken, for advice and assistance during the progress of the work and for a sympathetic understanding of the difficulties of the task undertaken.

UNIVERSITY OF MISSOURI,
COLUMBIA,
September 1, 1926.

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EXPLANATION OF PLATES.

All figures from the Duck are reproduced at the same scale. The drawings were outlined with an Abbe camera lucida at a magnification of 3,500 diameters, obtained with a Spencer 1/12 homogeneous immersion objective and a Zeiss 18x compensating ocular with draw tube set at 150 mm. and drawing made at the level of the base of the microscope. The drawings were then enlarged by means of a copying camera lucida to 7,350 diameters. Having been reduced one half in the reproduction, they now appear at a magnification of 3,675 diameters.

W, the large sex-chromosome carrying female-tendency genes only.

w, the smaller sex-chromosome which carries likewise female-tendency genes only.

Z, sex-chromosome carrying a preponderance of male-tendency genes and also sex-linked genes.

cr, crescent-shaped autosomes No. 32.

38Z, same as Z.

38w, same as w.

37 to 1, autosomes.



PLATE I.

FIGS. 1 to 6. Cells from the amnion of males of the Indian runner duck. The Z or sex-chromosome is numbered 38Z in each of the cells. 76 chromosomes present.

FIG. 1. Late prophase. The large chromosomes are shown in the characteristic peripheral position with the smaller ones within the circle.

FIG. 2. An earlier prophase than 1.

FIGS. 3, 5, 6. Cells in metaphase. The stippled line in each case indicates a possible grouping of the chromosomes in maternal and paternal groups. This grouping in each case has been considered with especial reference to the 12 largest chromosomes. The autosomes paired from 37 to 33, according to size.

FIG. 4. An early metaphase. Autosomes paired from 37 to 23.



FIG. 1



FIG. 2

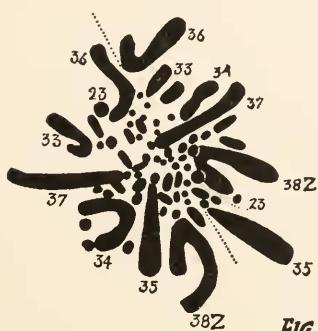


FIG. 3



FIG. 4

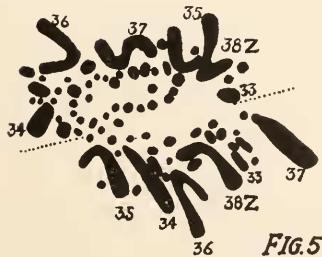


FIG. 5

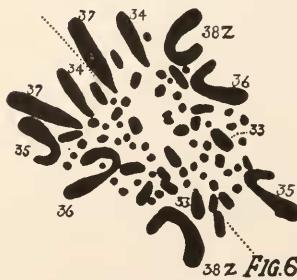


FIG. 6

PLATE 2.

FIG. 7. Metaphase of a cell from the amnion of a male Indian runner duck. The sex-chromosome is numbered 38Z. Autosomes from 37 to 33 are paired according to their size.

FIGS. 8 to 12. Cells from amnion of females of Indian runner duck. W and 38w are the chromosomes that are supposed to carry female-tendency factors only. Some filamentous linkage among the smaller chromosomes is shown in each cell. Autosomal pairs, numbered from 37 down, indicate their relative size in decreasing series. 77 chromosomes present.

FIG. 8. Prophase. Autosomes numbered from 37 to 33. Autosomes 29 are here designated *cr* to indicate their crescent form.

FIG. 9. Late prophase. Autosomal pairs from 37 to 1 indicated. The two groups of five small chromosomes each are shown at the upper left side of the cell.

FIG. 10. Prophase. Autosomes from 37 to 33 numbered.

FIG. 11. Prophase. The place of sutures in No. 37 is well indicated by bendings of the chromosomes in this cell. Autosomes from 37 to 33 numbered.

FIG. 12. Prophase. W in this cell is somewhat foreshortened.

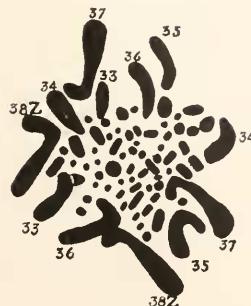


FIG. 7

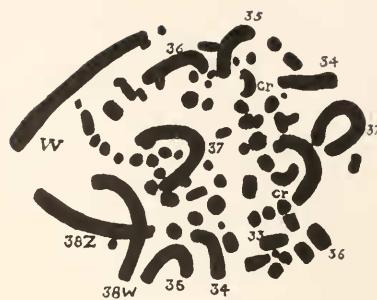


Fig. 8

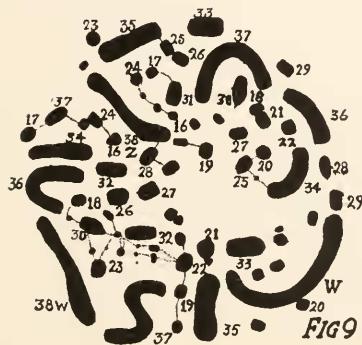


FIG 9

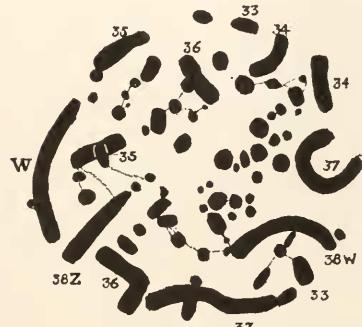


FIG 10

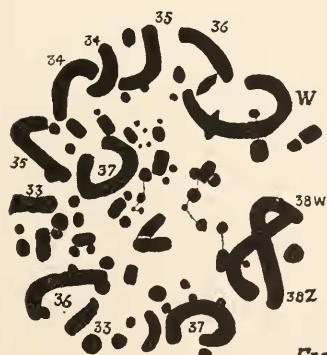


FIG. II

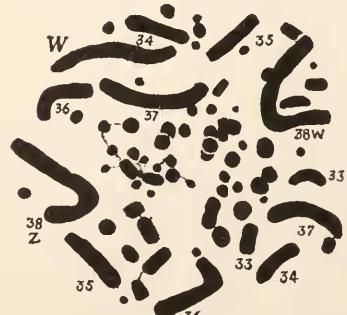


FIG 12

PLATE 3.

FIGS. 13 to 18. Cells from the amnion of females.

FIG. 13. Prophase. Autosomes from 37 to 33 numbered.

FIG. 14. Prophase. This cell is atypical in the arrangement of the large chromosomes. However, 77 chromosomes are present.

FIG. 15. Prophase. Autosomes 37 to 33 paired.

FIG. 16. Prophase. Autosomes from 37 to 1.

FIG. 17. An early metaphase. The W chromosome is bent upon itself.

FIG. 18. A late metaphase showing especially gonomeric grouping. The most of the large chromosomes in this cell are foreshortened.

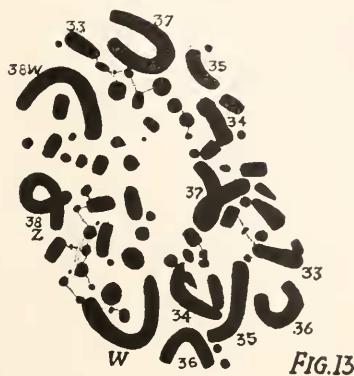


FIG.13



FIG.14

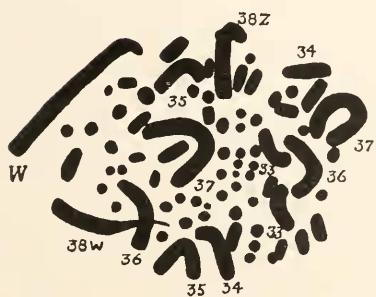


FIG.15

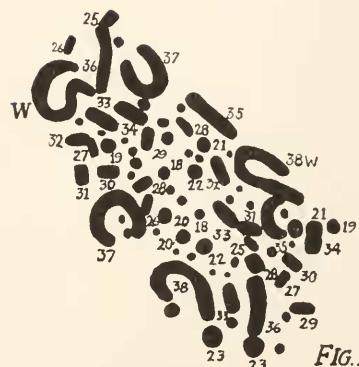


FIG.16



FIG.17

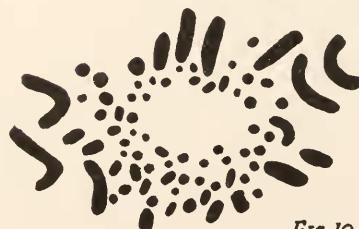


FIG.18

PLATE 4.

FIGS. 19 to 22. Cells from the amnion of females showing gonomeric grouping of the chromosomes.

FIG. 23. This figure shows some of the forms assumed by chromosome number 37; also the location of the sutures. Figures a, b, c, d are from cells of the male, and figures e, f, g, h, i are from cells of the female.

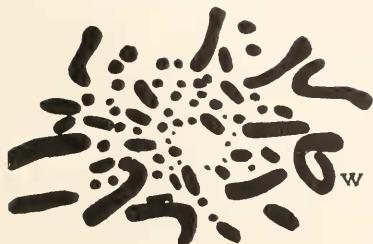


FIG. 19

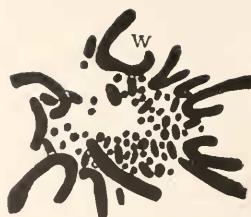


FIG. 20

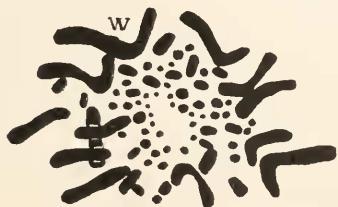


FIG. 21

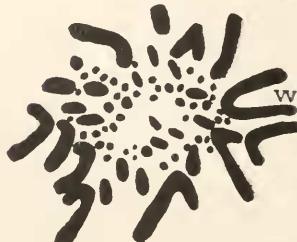


FIG. 22

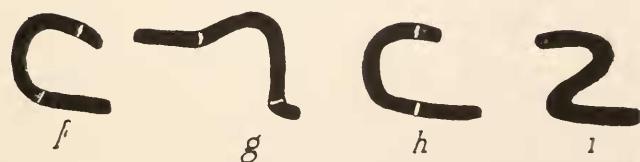
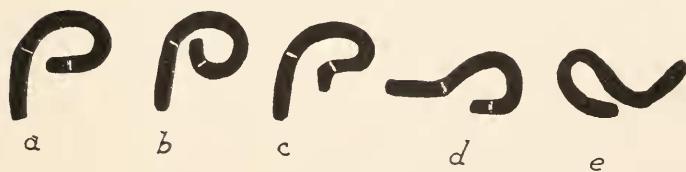


FIG. 23

PLATE 5.

FIGS. 24 to 29. First spermatocytes from smear preparations. The Z-chromosome bivalent in each cell is numbered 38Z, the autosomal bivalents from 37 to 1 according to their size. The cells are in the prophase stage.

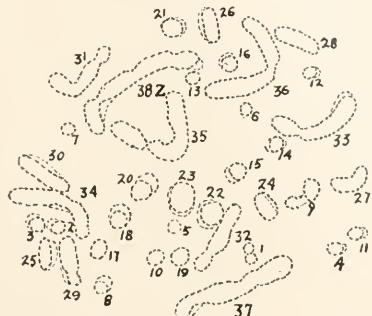


FIG. 24



FIG. 25

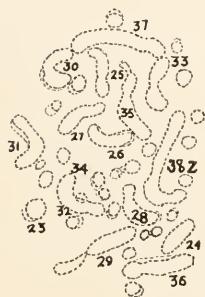


FIG. 26



FIG. 27

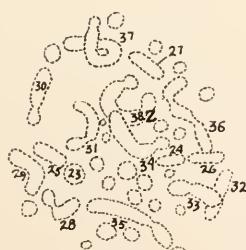


FIG. 28

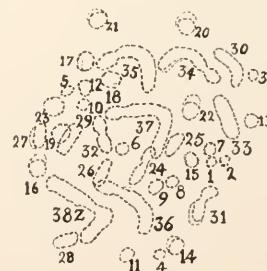


FIG. 29

PLATE 6.

FIG. 30. Chromosome complex taken from embryonic female gonad of chick. After Hance. In the original drawing the large chromosome at the top of the cell is labeled A to indicate that it is the largest in the complex. It is not paired in the cells of the female. I have added the line to the drawing to indicate a possible gonomeric grouping.

FIG. 31. Chromosome complex taken from a cell in an embryonic male gonad of the chick. After Hance. In the original drawing the two largest chromosomes at the top of the cell are labeled A, a, to indicate that they are the largest in the cell. They are thus present in duplicate in the male. I have added the line to indicate a possible gonomeric grouping.

FIG. 32. Somatic cell from the female of domestic chicken showing similar grouping. After Schiawago. The lettering is according to Schiawago and is his indication of the "paternal" (right) and "maternal" (left) grouping of the chromosomes.

FIG. 33. Somatic cell from the male of domestic chicken showing same. After Schiawago. The indications are as in figure 31.

FIG. 34. Spermatogonia cell of domestic chicken. After Stevens. The 12 large chromosomes are arranged in peripheral position. I have drawn no line here to indicate gonomeric grouping but such grouping is obvious.

FIGS. 35 to 36. Somatic cell from embryo of lizard (*Sceloporus spinosus*). After Painter. I have again added lines to each of the drawings to indicate possible gonomeric grouping.

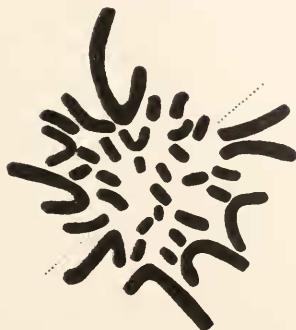


FIG.30

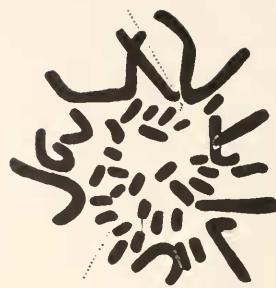


FIG.31

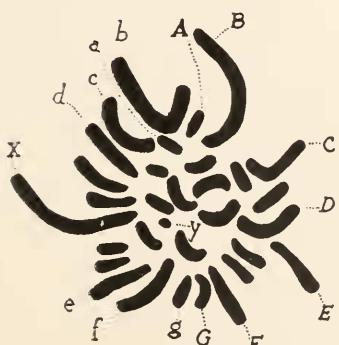


FIG.32

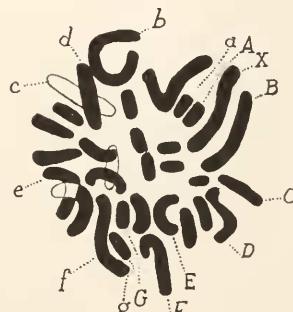


FIG.33

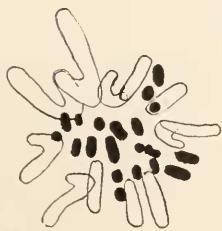


FIG.34

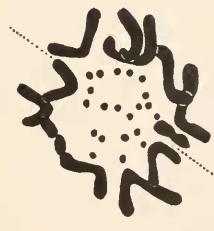


FIG.35



FIG.36

PLATE 7.

FIGS. 37 to 42. Chromosomes from the amnion of females artificially arranged according to size. W, w indicates chromosomes carrying female-tendency genes only. 38z indicates the chromosomes carrying sex-linked factors and supposedly a preponderance of male-tendency genes.

FIG. 37. Chromosomes from a prophase shown at Fig. 8. Autosomes 32 are here crescent-shaped.

FIG. 38. Chromosomes from a late prophase shown at Fig. 9.

FIG. 39. Chromosomes from a prophase shown at Fig. 13.

FIG. 40. Chromosomes from a prophase shown at Fig. 11.

FIG. 41. Chromosomes from a prophase shown at Fig. 12.

FIG. 42. Chromosomes from a prophase shown at Fig. 16.

W 38^w 38Z 37 37 36 36 35 35 34 34 33 33 32 32 31 31 30 29 28 27 26 25 24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2

FIG. 37

לְהַלְלֵךְ

FIG. 38

לְהַלְלֵךְ

FIG. 39

לְהַלְלֵךְ

FIG. 40

לְהַלְלֵךְ

FIG. 41

לְהַלְלֵךְ

FIG. 42

לְהַלְלֵךְ

PLATE 8.

FIGS. 43 to 48. Chromosomes from the amnion cells of males artificially arranged in pairs according to their size. 38Z, 38Z indicate what are probably the sex-chromosomes. Numbers 37 to 1 are probably autosomes.

- FIG. 43. Chromosomes from an early metaphase shown at Fig. 4.
- FIG. 44. Chromosomes from an earlier metaphase shown at Fig. 7.
- FIG. 45. Chromosomes from a metaphase shown at Fig. 3.
- FIG. 46. Chromosomes from a prophase shown at Fig. 1.
- FIG. 47. Chromosomes from a late metaphase shown at Fig. 6.
- FIG. 48. Chromosomes from an earlier prophase shown at Fig. 2.



FIG. 49



FIG. 50



FIG. 51



FIG. 52



FIG. 53



FIG. 54



PLATE 9.

FIGS. 49 to 54. Chromosomes from first spermatocytes from smear preparations. The Z bivalent chromosome is numbered 38Z. The autosomal bivalents from 37 to 1. All the cells are in the prophase stage.

- FIG. 49. Chromosomes from cell shown at Fig. 24.
- FIG. 50. Chromosomes from cell shown at Fig. 27.
- FIG. 51. Chromosomes from a cell shown at Fig. 29.
- FIG. 52. Chromosomes from a cell shown at Fig. 25.
- FIG. 53. Chromosomes from a cell shown at Fig. 28.
- FIG. 54. Chromosomes from a cell shown at Fig. 26.

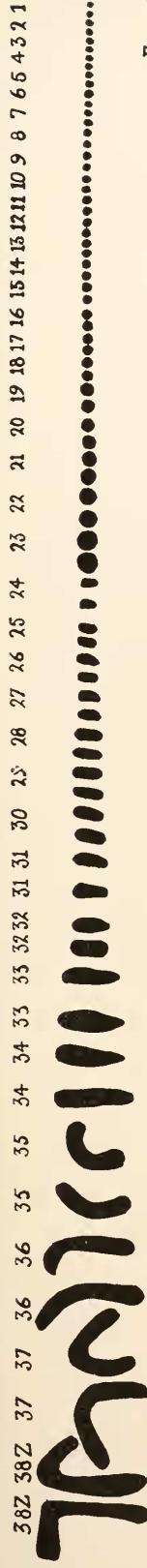


PLATE 10.

FIGS. 35 and 36. Scheme for sex-determination and sex-linkage in the Indian runner duck.

A indicates autosomes.

Z (double lined) indicates sex-chromosome carrying a dominant sex-linked gene, also carrying a preponderance of male-tendency genes.

Z (single lined) indicates sex-chromosome carrying a recessive sex-linked gene, etc.

W indicates the large sex-chromosome carrying female-tendency genes only.

w indicates the smaller sex-chromosome which carries likewise female-tendency genes only.

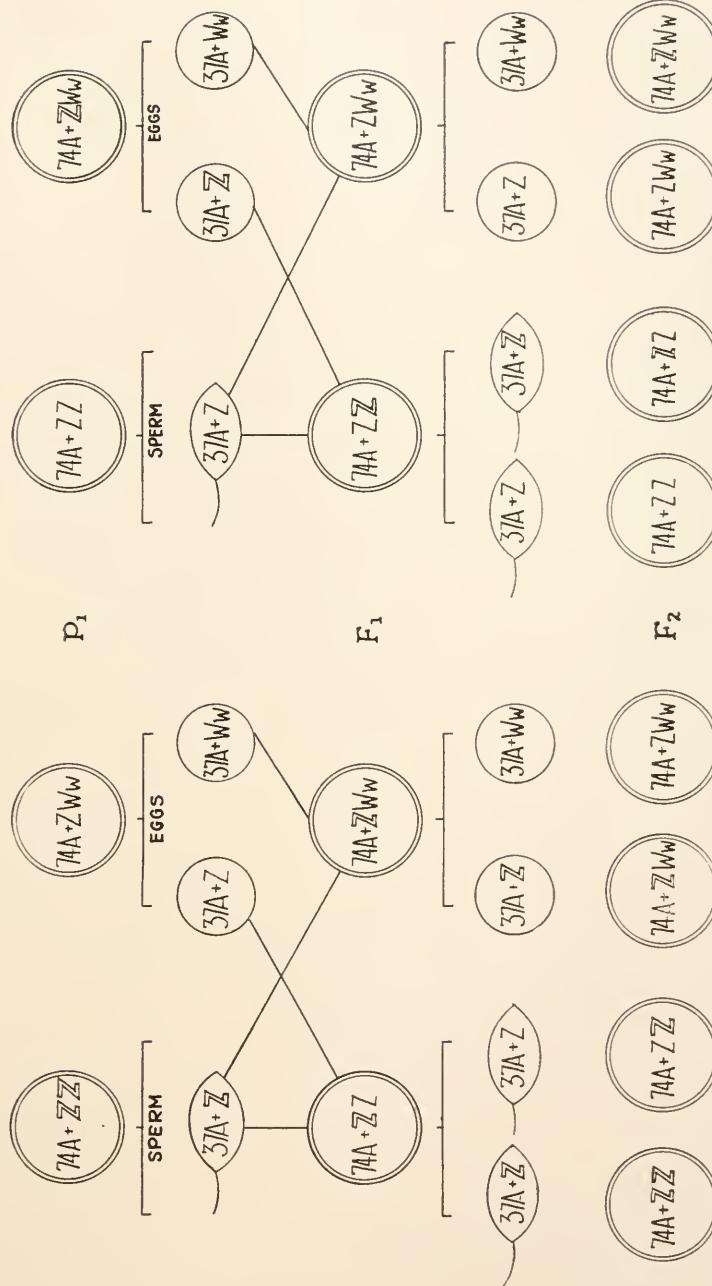


FIG. 55

FIG. 55

DEFECTIVE *PLUTEUS* LARVÆ FROM ISOLATED
BLASTOMERES OF *ARBACIA* AND
ECHINARACHNIUS.

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ORGANIZATION IN THE SEA URCHIN'S EGG.

Ever since Driesch in 1891 announced that a single blastomere of the two cell stage of the sea-urchin's egg separated by shaking would develop into a whole larva of half size, this method has been generally used by embryologists for determining the type of organization present in various eggs at the time of the first or later cleavages. Driesch's conclusions led to much controversy, but most investigators have come to a fairly definite agreement on the major facts to be learned from this field of investigation, and have turned to other problems. The recent important work of Von Ubisch (1925) has once more drawn attention to the organization of the sea-urchin egg previous to cleavage, and has made it necessary to revise certain accepted ideas. The data which I shall present appear to be of significance in this connection, and even though at this late date it would seem impossible to add anything to the facts regarding the development of isolated blastomeres of sea-urchin eggs, this will be my excuse for briefly reviewing the evidence.

In 1900 and in 1906 Driesch repeated and amplified his earlier work and stated his conclusion that in the sea urchin egg there was no evidence of differentiation at any cleavage stage, and that the egg was an harmonic equi-potential system, each cell of which might give rise to any part of the whole larva. In 1901 Boveri studied the normal development and later isolated blastomeres, and concluded that there was an entoblast zone below the equator of the egg and at right angles to its polar axis (colored red in *Paracentrotus*), without some portion of which an isolated blastomere would not develop an archenteron. He believed that

the first two cleavage planes cut this differentiated zone at right angles, and that the first cleavage plane marked the median plane of the larva. In support of this idea he pointed to the fact brought out by Driesch himself that the isolated animal half of the transversely divided eight cell stage seldom gastrulated, while the isolated basal half (vegetative portion) more often developed normally. Driesch did not accept this interpretation, and in 1906 described the development of an egg incompletely separated in the two cell stage but differentiating as one individual. Here he found that the skeletal rudiments appeared in the descendants of one only of the original cells, and he concluded therefore that the first cleavage plane cut at right angles to the median plane of the larva, and that, contrary to Boveri, the egg was first divided into animal and vegetative portions.

Here the matter has stood up to the present. The majority opinion has accepted Boveri's evidence, as shown by Conklin's summing up of the situation (1924, page 586): "It is plain that there is a differential distribution of egg substances to the cleavage cells from the animal to the vegetative poles, though no differentiation of cells in any cross axis can be detected until much later." E. B. Wilson (1925, page 1067) said: "These facts (e.g., of Boveri) demonstrate that the sea-urchin egg is no more isotropic than that of the mollusk or annelid."

The experiments of Von Ubisch mentioned above prove that both Driesch and Boveri were right with respect to the relation of first cleavage plane and the median plane of the embryo, yet in spite of this are in no sense incompatible with Boveri's idea of a differential organization of the egg at right angles to the initial egg axis. This investigator has succeeded, where Boveri failed, in staining one or more of the blastomeres in dividing eggs of *Echinus* and *Echinocyanus* and then noting the portions of the larvae which are marked with the dye. By this method he has demonstrated quite conclusively that there is no constant relation between the first cleavage plane and the median plane of the larva, since the stained area may appear in any region of the pluteus. Thus larvae have been found which differentiated both as described by Driesch and by Boveri, and at every angle between. In spite of this fact Von Ubisch believes that his results

lend support to Boveri's conception that the egg is stratified at right angles to the initial egg axis. With such a distribution of differentiated substances and the first cleavage cutting at any angle, one would expect that staining one blastomere of the two cell stage would give larvæ with the blue area in every possible sector. This Von Ubisch found. Such a result obviously is not at variance with Boveri's view, but it can hardly be called evidence in favor of it for the same might be true if there were no organization of the egg whatever. The only disproof of the latter idea would consist of the demonstration of the same sort of relation in larvæ actually developed from isolated one half blastomeres. This evidence I have secured for *Arbacia* and *Echinorachnus* as will be indicated below.

In addition to the problem of the organization of the undivided egg of the sea urchin with respect to stratification of differentiated substances, there is the added question as to a possible bilateral organization of such materials. Boveri obviously held that the right and left sides of the body of the larva were determined by the plane of the first cleavage, but Von Ubisch's work disproves this. The same criticism applies to the conclusions of Schaxel (1914) and of others who worked with isolated blastomeres. If bilaterality bears no relation to the first cleavage plane, then it must be determined in the egg before the first cleavage or at a much later stage in development. To establish the first relation, since the first cleavage cuts the egg at any angle, it would be necessary to show that isolated blastomeres formed only certain definite parts of some obviously bilateral structure like the skeleton. It seems extremely unlikely, and yet curiously enough there is good evidence for exactly this situation in at least one sea-urchin, *Paracentrotus*—the same egg, by the way, in which appears the stratified pigment layer described by Boveri. J. Runnström (1914) described the development of isolated blastomeres of this egg, and for some reason his results seem to have been largely overlooked in discussions of the subject. Quite contrary to the results of Driesch on *Echinus* and *Sphaerechinus* he found that the blastomeres of the two cell stage did not develop as whole larvæ even after the blastula stage. Instead they gave rise to partial larvæ, showing the skeleton of either the right or

the left side. Such larvæ never become normal. Unfortunately data are not given to show how many blastomeres developed in this manner, nor what happened to the other member of the pair when one gave this result. Apart from this, however, Runnstrom's conclusion seems justified that there was an indication of a bilateral organization of skeleton forming material in the undivided egg of *Paracentrotus*. I have found a number of cases of the same sort in *Arbacia* and *Echinorachnius*, suggesting that in these eggs also there is already some bilaterality of skeleton forming material in the undivided egg.

The bearings of the present investigation may be shown with greater clearness by reference to a diagram. Several of the earlier writers have indicated that material which goes to form the larval skeleton seems to be localized in the four micromeres cut off from the lower quartette of cells at the fourth cleavage (sixteen cell stage). Von Ubisch has apparently confirmed this by staining these cells and finding that the material derived from them formed the larval mesenchyme. On Boveri's view this skeleton forming substance may be thought of as stratified at right angles to the initial egg axis in the undivided egg. That this is probably the case in *Arbacia* is indicated by the recent work of Harnly (1926). He reported experiments in which fertilized eggs of *Arbacia* were cut in two before the first cleavage, and he found that the nucleated portions segmented either as the dorsal, lateral or ventral hemisphere as determined by the presence or absence of micromeres. This result shows, he believes, that there is a localized equatorial area of micromere forming substance in the undivided egg below the nucleus and in the vegetative half of the egg. The later history of these fragments is not described, but we may assume that the material which will form the skeleton is located in the egg as suggested. According to Von Ubisch's evidence the first cleavage plane may cut the egg as shown in Fig. 1. *A* and *C* indicate the extremes, and *B* one of the possible planes between. According to this plan we should expect that each blastomere of *A*, if isolated would develop as a whole larva of one half size. If those of *C* are separated, however, the upper blastomere would lack the skeletal material, and one complete larva and one lacking a skeleton

might be expected. Finally if one of the many eggs in which the cleavage fell between the two extremes, like *B*, was operated on, either the same result or two larvae with defective skeletons might be looked for. The possibility of bilateral organization

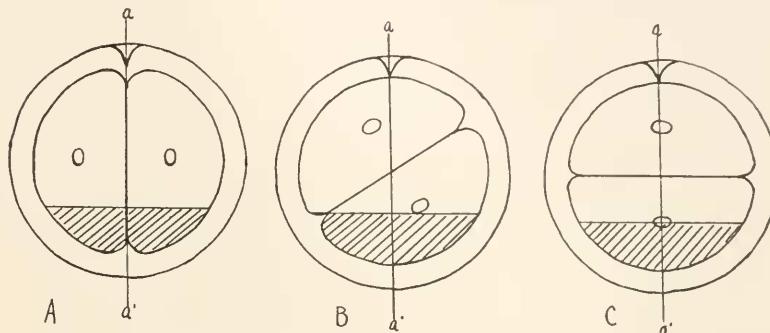


FIG. 1. Diagrams showing three possible relations of the first cleavage plane to the primary egg axis as indicated by the micropyle. The line *a-a'* is drawn through the egg axis in each case. The shaded layer suggests the possible location of the skeleton forming material.

need not be considered for the present. In any case the results of the development of isolated blastomeres, would seem to offer conclusive evidence as to the differentiation in the sea-urchin egg in the light of the work of Von Ubisch.

Considered from this point of view it becomes an important matter to secure data on the later development of a large number of isolated blastomeres of sea-urchin's eggs. Such data must record the complete results and not simply the fact that complete larvae of one half size are formed from isolated blastomeres of the two cell stage. It is obviously quite as important to know how many such blastomeres do not form complete larvae, as to know how many do. Practically all of the earlier investigators note that a number of failures occur, but they are content with the positive demonstration. Even under the best conditions and with the greatest care in handling however, a certain number of whole eggs fail to develop normally, and this is sure to be true to an even greater extent when the cells are subjected to the handling required by the technique of separation of the blastomeres. This is true even when the earlier stages of cleavage are normal. Data of the sort suggested therefore can not be

conclusive, for one can never be certain how many of the failures are due to deficiency of materials in the initial cell and how many to abnormal development resulting from the handling.

This difficulty in interpreting the results can be eliminated to a very large degree if the development of both members of the pair of blastomeres from one egg are followed, and the results recorded for single pairs rather than for groups of cells. When one or both of the blastomeres fail to develop to the stage of the ciliated larva, this pair need not be considered. When, under the same conditions and with identical handling, both blastomeres do develop to this stage the result may be considered significant evidence of organization of the undivided egg. It seems an extraordinary fact that in all of the published work on isolated blastomeres of sea-urchins there is so little in which the developmental history of each of the blastomeres derived from a single egg is known. In all of his earlier work Driesch was content to show that whole larvæ of one half size were formed from isolated one half blastomeres, and no attempt was made to prove that both blastomeres from one egg did so, nor even to indicate how many out of a large group failed to differentiate in this way. In 1906 Driesch published his first study of the "Physiology of Bilaterality," in which he did take up this question. He followed the development of both blastomeres of the two cell stage, usually two eggs at a time, in several series of experiments on *Echinus multituberculatus* and *Spærechinus*. The eggs were observed for two days only, and failures were not recorded. From this data I find that out of 9 eggs divided, only 2 gave normal one half size plutei from both blastomeres, 4 gave two "prismen" larvæ—too young to determine whether the skeleton was normal—2 from which one pluteus was complete and the other apparently incomplete, and 1 in which no skeleton appeared in either. These data are insufficient for any conclusion as to the organization of the egg along the lines indicated, yet so far as they go they suggest that both blastomeres do not necessarily develop into whole larvæ. In all of Driesch's remaining experiments, including several series reported in his 1908 paper on the same subject, the blastomeres were observed for one day only—that is to the gastrula stage—and it was assumed that they would develop into

normal larvæ if they formed normal gastrulæ. This assumption is quite unjustified, as my data on *Arbacia* show.

PRELIMINARY EXPERIMENTS.

Several years ago at the Marine Biological Laboratory I began experiments to determine whether in the egg of *Arbacia* both blastomeres isolated in the two cell stage would form normal half size larvæ. Each egg was handled separately, and the development was followed for at least four days. The work of the first season—1924—gave so few pairs of blastomeres each of which developed that the point could not be settled. At the same time it became apparent that one half blastomeres give rise: (1) to ciliated larvæ which either do not gastrulate or do not form a skeleton, (2) to larvæ with incomplete or partial skeletons, and (3) in a small number of cases to whole larvæ of one half size. These facts I reported briefly at the Washington Meeting of the Society of Zoologists in 1924.

During the past two seasons at Woods Hole I have been able to extend these observations on *Arbacia*, and during 1926 I made a similar series on the egg of the sand dollar *Echinorachnius*. The latter egg is much more favorable for such work, since it is larger, has a membrane which is much less resistant, and when good lots of eggs are obtained, gives a high percentage of eggs which develop into normal plutei. It is harder to get good lots of eggs of *Echinorachnius* than of *Arbacia* at Woods Hole, and therefore the total number of blastomeres followed is less.

METHODS.

The eggs were removed from the animals into sea water, washed, and fertilized with motile sperm suspensions. During the earlier work with *Arbacia*, the membranes were removed by shaking a suspension of eggs in a small test tube for twenty seconds at an interval of one to one and a half minutes after fertilization. This violent treatment is necessary to break and remove the very tough membrane of *Arbacia*, and it generally breaks up or otherwise injures a large number of the eggs. It was later found possible to remove the membranes with great ease, either about fifteen minutes after fertilization or in the two

cell stage, by sucking them up into a very fine capillary pipette with a bore about two thirds the diameter of the egg membrane. If the pipette is of the proper size, and the eggs drawn up carefully by means of an attached rubber tube held in the mouth, this method seldom injures the eggs. It was used throughout the larger part of the work. When the eggs had passed into the two cell stage, a small number were picked up with a slightly larger pipette and placed in a few drops of sea water on a clean glass slide.

The blastomeres were separated with fine glass needles used free hand under a binocular microscope. It is possible to use either one or two such needles, and with practice to acquire a high degree of skill in their use. Needles for this work must be fine enough to lie easily in the furrow between two cells, and stiff enough to allow of a small amount of pressure. Ordinarily needles which are sufficiently fine, work better if they are not too long—about ten times the diameter of the egg seemed to give the most satisfactory results. This free hand method has been used recently by a number of investigators for work in cutting eggs in preference to the more rigid and much slower microdissection apparatus. (Cf. especially Fry 1924, who has given a complete account of the method of making the needles and their use.) It has the advantage that many more eggs can be operated on in a given period, and with practice the control is quite as dependable. The needle is laid along the furrow between the blastomeres, and drawn gently back and forth. Usually *Arbacia* blastomeres can be separated cleanly and without injury by one or two strokes. Often the egg sticks to the needle, and rolls with it, but if this is not prolonged it seldom results in injury. In some cases the blastomeres will separate the width of the needle, but no further, remaining attached apparently by a thin bridge of protoplasm. Such eggs were usually discarded, although the blastomeres seldom come in contact again and eventually separate.

The two cell stage of *Arbacia* with the membrane removed exhibits certain differences from the normal which are of importance in separating the blastomeres. Shortly after division the two cells round out, forming two nearly perfect spheres having a very narrow area of contact. They remain in this condition

for fifteen or twenty minutes, gradually flattening against each other as the next division approaches, until at the time of the second cleavage they appear as half spheres with their flattened diameters in contact. The same process occurs at each cleavage, and is apparently caused by the gradual recurrent increase in viscosity up to the time of actual division. The blastomeres must be separated during the earlier period when they are sphere shaped, since it becomes increasingly difficult later.

I have made many attempts to separate the blastomeres of *Arbacia* by the use of Ca-free sea water, so widely used for this purpose since the original discovery of the method by Herbst but all of these have been unsuccessful. The original Herbst solution used at Naples, sea water treated with Na-citrate, and various artificial sea waters without Ca, have uniformly failed to bring about the separation of *Arbacia* blastomeres without shaking, and this makes it impossible to keep the two blastomeres of one egg under observation. With *Echinorachnus* the method seems to be somewhat more successful, but not uniformly enough to be superior to mechanical separation.

The isolated blastomeres were picked up one by one with a capillary lip pipette, and the pairs from any one egg placed together in sea water in a round bottomed glass dish two or three centimeters in diameter with a slot ground in the center (Lefevre dishes). These dishes were more satisfactory than depression slides or other containers, since by placing the blastomeres in the slot the water can be changed without danger of sucking up the cells. The development was observed under the four millimeter objective of the compound microscope with a ten ocular.

The dishes when not under observation were kept in a moist chamber consisting of a glass evaporating dish with a cover. This was kept in the tank with running sea water constantly passing over it to maintain it at approximately the temperature of the sea water itself. The water in each dish was changed daily, actual tests showing that a negligible change in hydrogen ion concentration occurred during this period.

In general the cleavage stages of all separated blastomeres were followed and sketched. After that the embryos were observed at least once a day up to the fifth day. Several control eggs with

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the membranes removed were placed in similar dishes with each series of experiments.

EXPERIMENTS.

Summary of All Isolated Blastomeres.—In Table I. a tabular view of all of the results is given, regardless of whether or not both members of the pair of isolated blastomeres developed. All blastomeres which began cleavage normally are included. In the last three columns the figures in brackets are the percentages of the number which lived to the ciliated larva stage.

TABLE I.

(1) Number of Blastomeres Isolated.	(2) Total Number of Gastrulæ.	(3) Ciliated Larvæ with No Skeleton.	(4) Pluteus with Defective Skeleton.	(5) Complete Pluteus of $\frac{1}{2}$ Size.
<i>Arbacia</i> , 268 <i>Echinorachnius</i> 33	121 20	46 (47 %) 5 (31 %)	24 (24 %) 6 (38) %	28 (29 %) 5 (31 %)

The summary indicates that a large number of isolated blastomeres even when apparently uninjured, do not develop normally. Less than 50 per cent. of the *Arbacia* cells developed to the gastrula stage, although the number in the sand dollar was larger. Of the isolated cells which lived to become ciliated larvæ,

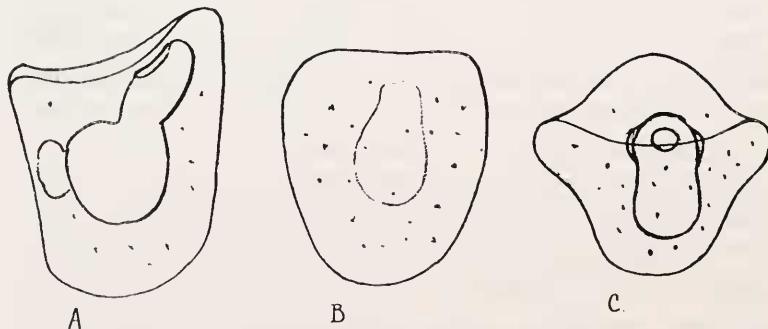


FIG. 2. Ciliated larvæ with no skeletons developed from single blastomeres of the two cell stage of *Arbacia*. Four days old. Measured and drawn free hand from living specimens to the same scale.

47 per cent. in *Arbacia* and 31 per cent. in the sand dollar never developed a skeleton. Doubtless a certain number of these were

injured and therefore failed of normal development, but in view of the evidence given in the next table where the development of pairs from the same egg are listed, it is probable that some of them were deficient in cytoplasmic material already localized at

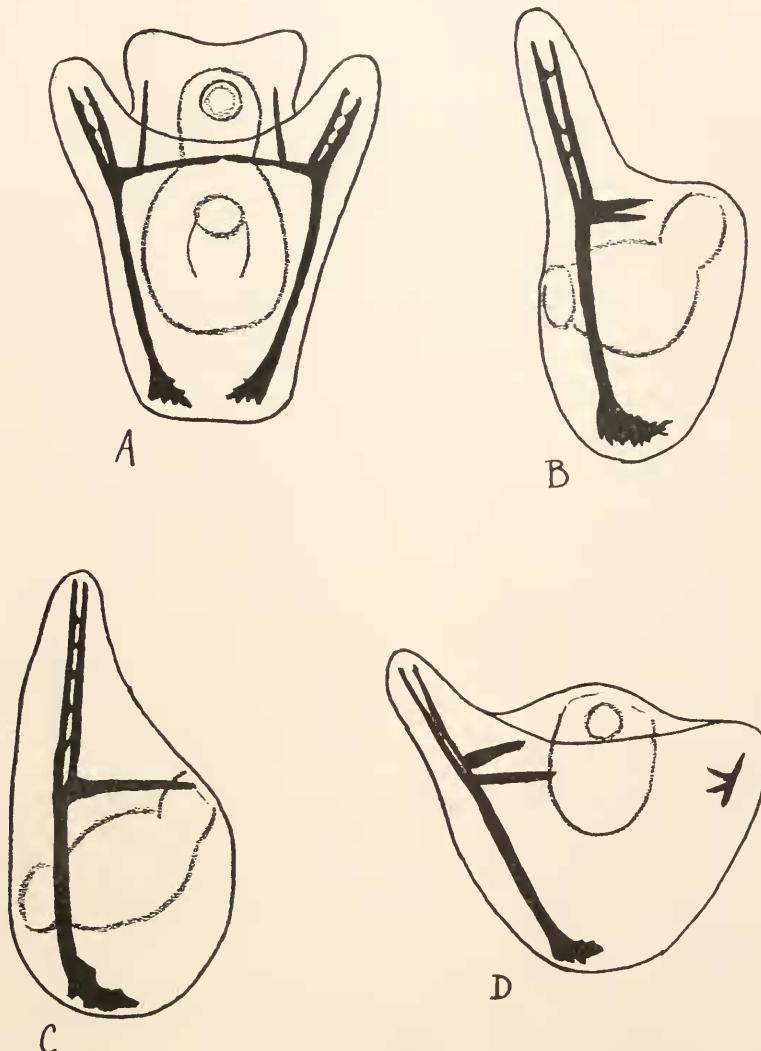


FIG. 3. Pluteus larvæ four days old developed from single blastomeres of the two cell stage of *Arbacia*. *A* is a larva with a complete skeleton of approximately one half size. *B*, *C*, and *D* are larvæ with defective skeletons. Measured and drawn free hand to the same scale from living specimens.

the time of the operation which later goes to form the skeleton. Many of these larvæ lived for eight days or even more, but always remained enlarged ball-like larvæ (Fig. 2). In perhaps half of these larvæ a normal tripartite gut was formed, while in the others either a small portion or none at all was differentiated. Some of these larvæ were probably derived from blastomeres like the upper one in Fig. 1 C.

The larvæ listed in the fourth column of Table I. are of special interest. They were briefly described in my preliminary report. All showed the development of a skeleton but this was incomplete. The commonest type was a four day pluteus with one long postoral arm and the other absent. The oral portion of the skeleton was usually missing entirely. Fig. 3 shows several such larvæ compared with a complete one half size larva of the same stage. Such larvæ of *Paracentrotus* have been figured by Runnström (1914) in earlier stages. It seems impossible to escape the impression that such larvæ are derived from one of the blastomeres of an egg divided like B in Fig. 1.

The larvæ listed in the last column of Table I. were normal but of approximately one half size (Fig. 3). They were complete in every observable detail, and were usually as viable as normal control plutei. Such larvæ are probably derived from cells divided along the egg axis (Fig. 1 A). It was observed that isolated blastomeres developed at a slower rate than normal control eggs. The first cleavage appeared fifteen to twenty minutes after the second in the normal egg, and the gastrulæ were sometimes two hours or more behind.

Finally it should be stated that I have never found larvæ with defective skeletons among the normal controls unless these were subjected to abnormal treatment. Shaken eggs sometimes give as many as 15 per cent. of abnormal skeletons, but never eggs handled as indicated. Almost every group of control eggs shows a few larvæ which do not gastrulate, or which do not form skeletons, but these seldom live more than two days and usually less.

PAIRS OF BLASTOMERES FROM THE SAME EGG.

If the results summarized above seem inconclusive because of the large number of blastomeres which fail to develop to the

pluteus stage, it seems possible to attain to a fair degree of certainty by limiting our consideration to those eggs only of which both blastomeres developed to the stage of a ciliated larva. These data I have tabulated in Table II.

TABLE II.

SHOWING TYPES OF LARVÆ RESULTING FROM THE TWO BLASTOMERES OF THE SAME EGG, WHEN BOTH LIVE TO THE CILIATED STAGE.

(1) Number of eggs Separated	Pairs of Separated Blastomeres Gave rise to					
	(2)	(3)	(4)	(5)	(6)	(7)
2 Ciliated Larvæ with No Skeleton.	1 Larva No skeleton and 1 Complete Pluteus.	1 Larva No skeleton 1 Defec- tive Pluteus.	2 Defec- tive Plutei.	1 Defec- tive Pluteus, 1 Com- plete Pluteus.	2 Com- plete Plutei.	
<i>Arbacia</i> , 18 (100 %)	8 (44 %)	5 (28 %)	2	1	1	1
<i>Echinorachnius</i> , 9 (100 %)	1 (11 %)	3 (33 %)	1 17 % 45 %	3	11 % 0	11 %
Representing probable relation of plane of first cleavage to egg axis as in:	?	Figure 1 C	Figure 1 B		Figure 1 A	

Table II. is largely self-explanatory. The total number of eggs of which both blastomeres developed is only 7 per cent. of the total number of blastomeres followed in *Arbacia*, but is 54 per cent. in the sand dollar. When good eggs of the sand dollar can be secured it is obviously a much more favorable species for such work. The same fact appears in column (2), since in 44 per cent. of the eggs of *Arbacia* which gave rise to two larvæ no skeleton developed in either, while only one out of nine, or 11 per cent. gave this result in the sand dollar. This fact introduces a source of error in the interpretation of columns (3) and (4), for it is not certain that some of the larvæ with no skeleton here might not have possessed skeleton forming material. This possible error is not serious, however, as an examination of the remaining data will show.

A study of the remaining columns in the table reveals three fairly striking facts. First, the number of pairs of blastomeres each of which form complete larvæ is very small indeed. I have found only one out of eighteen eggs which gave this result in *Arbacia*, and none out of nine in the sand dollar, although the one recorded in column (6) showed only a slight defect in one arm of one of the larvæ. Second, among such pairs larvæ with defective skeletons are fairly common, but occur most often in pairs, column (5). Probably the error shown by column (2) has prevented some of those listed in column (4) from appearing in (5). Third, there is a fairly large group of pairs of which one member formed one complete half size pluteus and the other a larva with no skeleton, column (3).

For the purpose of our analysis then there would appear to be three significant groups of eggs. While the actual numbers are small they form a selected group from a very much larger number, which as shown above tends to suggest the same result. The first group consists of the small number of eggs each of whose blastomeres formed perfect plutei, the second of eggs each blastomere of which formed an incomplete pluteus, and the third of eggs one blastomere of which formed a perfect pluteus and the other a larva with no skeleton. It is obvious that these three main groups correspond in general with the three possible directions of the first cleavage plane with reference to the initial egg axis as suggested by the work of Von Uebisch, and as indicated in diagrams *A*, *B*, and *C* in Fig. 1. This interpretation is noted in the last line of Table II. Not only are these three groups found, but in the sand dollar eggs at least the percentages of eggs which fall in them correspond with expectation. The second group should be the largest, and the first the smallest, as in fact they are.

DEFECTIVE PLUTEI AND THE EVIDENCE FOR BILATERALITY OF SKELETON-FORMING MATERIAL.

A study of the larvæ with defective skeletons not only substantiates the facts stated above, but suggests in addition a certain amount of bilateral organization of the skeleton-forming material which is apparently already localized in the vegetative half of the egg at right angles to the initial egg axis. All of the

larvæ listed in column (5), Table II., showed defective skeletons. Two sets of these—the *Arbacia* pair and one of the pairs from an *Echinarachnius* egg—were larvæ of the one armed type illustrated in Fig. 3. The other two pairs of *Echinarachnius* larvæ showed skeletons which were clearly complementary, that is the one member showed the parts which the other lacked. These two pairs of larvæ are shown at the age of four days in Fig. 4, A and

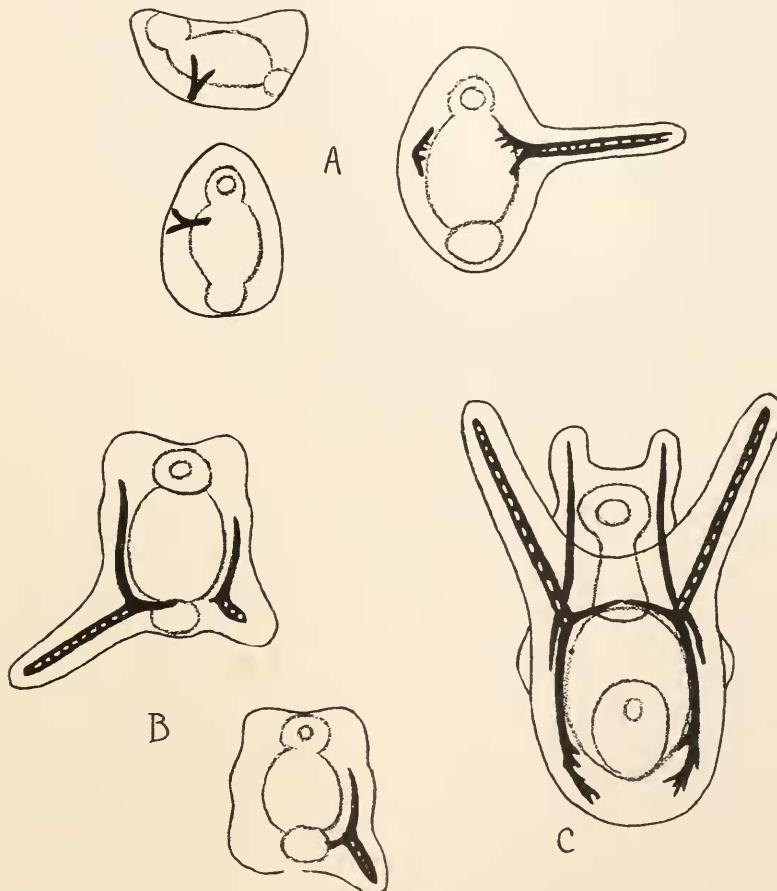


FIG. 4. Pluteus larvæ of *Echinarachnius* four days old. A shows two larvæ developed from the two isolated blastomeres of the two cell stage of the same egg, the smaller one shown from the side and the top, the larger from the top only. B shows a similar pair from the top only. C shows a normal control larva of the same age, aboral view. Measured and drawn free hand to the same scale from living specimens.

B, compared with a normal control larva of the same age in *C*. All the defective larvae had a complete tripartite gut like the normal. In the *A* pair one shows a skeleton like the control but with the large *left* postoral arm lacking, while the other has only a rudiment of a spicule on the left side—the *right* side completely lacking. These larvae were followed for two days longer and the latter never developed any farther, while the former never replaced the defect. In *B* much the same result is shown, perhaps more distinctly, but rather more skeleton appeared in the smaller larva. As indicated above Runnström (1914) described such larvae in earlier stages as occurring regularly in *Paracentrotus*, and suggested that the undivided egg showed a bilateral organization. The finding of these plutei in *Arbacia* and the sand dollar constitutes very clear proof that a localization of skeleton forming material has taken place in the undivided egg, and that this may be divided unequally by the first cleavage plane. It also indicates a bilateral organization of this material. It seems quite probable that the reason that this bilaterality is not more often evidenced is that blastomeres with a marked deficiency of skeleton-forming material often fail to develop the skeleton rudiment at all.

It may be asked in view of this interpretation how it happens that two complete plutei ever develop from both blastomeres of one egg. To this it may be answered that my data indicate that such cases are extremely rare in *Arbacia* and the sand dollar. When the stratified material is about equally divided apparently the initial bilaterality may be reorganized from the start. That such reorganization may occur infrequently even in later stages seems to be proved by the history of one *Arbacia* blastomere, in which the steps were noted with great clearness.

In the case illustrated in Fig. 5 one isolated blastomere began its development as a partial one, but later a complete but small skeleton was regenerated—or postgenerated. In this case both blastomeres of the egg went through the normal one half cleavage, becoming rounded up at the blastula stage. Two one-half-size gastrulae were formed, but one never became motile and soon died. The other apparently formed a single triradiate spicule and developed at the end of two days a half skeleton with a single postoral arm, and the crossbars as indicated in the sketch. On

the third day the skeletal material had extended over to the other side and a second postoral arm had appeared. The oral part of

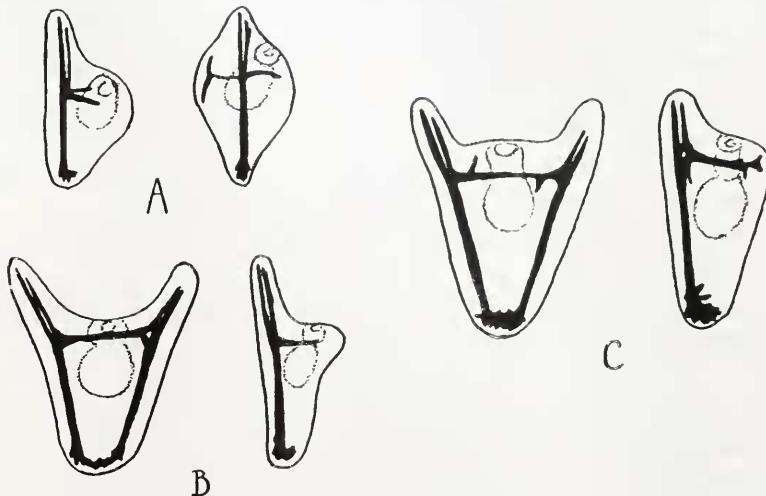


FIG. 5. Successive drawings of a pluteus larva developed from a single blastomere of the two cell stage of *Arbacia*. The skeleton was first one-sided, but later all except the oral portion was regenerated. A two days old, B three days old, C five days old. An aboral and a side view are shown in each case. Measured and drawn free hand to the same scale from the living specimen.

the skeleton was defective. By the fourth day a skeleton with all parts represented, but abnormally massive, and of rather less than the usual size was formed.

CLEAVAGE OF ISOLATED BLASTOMERES.

The work of Driesch, of Morgan, and others indicated that the cleavage of isolated blastomeres of *Echinus* and *Sphærechinus* was that of half embryos. Each blastomere divided into four equal cells, and at what corresponds with the fourth cleavage of the original egg gave rise to two micromeres, two macromeres and four mesomeres, just as they would have done in the normal egg. They later formed an open blastula with half the normal number of cells, which eventually rounded up. Since in the cleavage of *Echinorachnus* there are no clearly distinct micromeres, it is impossible to compare its cleavage with that of the others. The cleavage of the one half blastomeres of *Arbacia* was followed, but

because of the difficulty of being certain of all the cells when rapid cleavage was going on, and when a number of eggs are under observation at the same time, I have accurate records on only about twenty-four blastomeres of this type. Fifteen of these developed as stated above for *Echinus*, namely giving off two micromeres at the third cleavage (normal fourth). The others appeared to have formed no micromeres at all—rather eight equal cells—though two may have had one only. Only eight of the total number developed to the pluteus stage, a number far too small from which to draw positive conclusions. From half eggs showing two micromeres there developed larvæ both with and without a skeleton, and in two cases a larva with a skeleton developed from blastomeres in the cleavage of which no micromeres were observed. So far as they go the data suggest that there is no essential relation between the presence of micromeres in artificially divided eggs and the development of a skeleton. Yet Von Ubisch has shown that the skeletal material is normally passed out into the micromeres, and Harnly's work apparently indicates that when the vegetative portion of the undivided egg is cut off, micromeres do not develop. On the other hand Tennent and Taylor, working on another egg cut before fertilization, did get normal larvæ from fragments which formed no micromeres. It seems probable that normally the pattern of cleavage is determined by the first cleavage plane, and the skeleton forming material goes as usual into the micromeres. When the first two blastomeres are severed this pattern is ordinarily continued, but it may sometimes be disturbed. Whether it is or not apparently has no influence on the development of a skeleton, since that in *Arbacia* at least appears to depend on the presence or absence of skeleton forming material in either or both of the separated blastomeres.

Since this paper was written the more complete account of the work on development of fragments of the unfertilized egg of *Lytechinus* by Taylor, Tennent and Whitaker (1926) has appeared. Their work convincingly proves that in this egg micromere forming substance is not differentiated before fertilization. Whether their disagreement with the findings of Harnly is due to differences in behavior between *Lytechinus* and *Arbacia*, future work

may show. In any case their observations as to the variability in the number of micromeres agree with my own in the development of the severed blastomeres of the two cell stage of *Arbacia*. If the eggs are similar, it would seem to be indicated clearly that the localization of material which later forms the skeleton occurs between fertilization and the cutting through of the first cleavage.

DIRECT DETERMINATION OF THE RELATION OF THE EGG AXIS TO THE FIRST CLEAVAGE PLANE.

The interpretation indicated by the data presented above, namely that there is a stratified layer of skeleton forming material at right angles to the egg axis which may be cut at any angle by the first cleavage and unequally distributed to the first two blastomeres, is obviously susceptible of a direct test. It is possible to determine the egg axis, using the micropyle as a pointer, by the use of a suspension of Chinese ink in the sea water. The angle which the first cleavage plane makes with this axis can then be noted, the membrane removed, and the blastomeres separated. In practice however this is an exceedingly difficult and time-consuming operation. In *Arbacia* at least it is often difficult to make out the micropyle with certainty, and the large number of blastomeres which fail to develop to the larval stage after membrane removal and separation usually make the laborious determination valueless. I have not been able to carry it out in enough cases with *Arbacia* to make the results of any significance. The method was not tried with the eggs of *Echinarachnius*, which appears to be a more favorable species for this work. In future work I hope to make this determination in a larger number of cases.

The relation of the egg axis to skeleton forming or other material localized in the egg might be tested in the same way by Von Ubisch's method also. The primary axis might be determined, and with this actually in view, a sector of the egg at right angles to it might be stained with the purple dye. Von Ubisch has apparently not made this determination, probably because of the difficulties involved.

SUMMARY AND CONCLUSIONS.

1. The developmental history of more than three hundred blastomeres of *Arbacia* and *Echinorachnius* isolated in the two cell stage and followed individually for at least four days is recorded.
2. About forty per cent. of these blastomeres form ciliated arvae with a complete tripartite gut, but these larva fall in three classes with respect to the skeleton; (a) those which never develop a skeleton, (b) those in which the skeleton is defective or partial, (c) those with a complete pluteus skeleton of one half size.
3. When the pairs of blastomeres from the same egg both of which developed to the larval stage are considered alone, it is found that they fall into three significant groups: (a) a very small number of pairs from which developed two plutei with perfect skeletons of one half size, (b), a much larger number from which developed two plutei each with a skeleton which was incomplete, (c), a group which formed one perfect pluteus and one larva without a skeleton.
4. These facts appear to prove that there is already localized at the time of the first cleavage in the eggs of *Arbacia* and *Echinorachnius* a layer of skeleton forming material at right angles to the primary egg axis, and in the vegetative half of the egg, which may be cut at any angle by the first cleavage plane and unequally distributed to the first two blastomeres.
5. The eggs of these two sea-urchins are thus shown to be (to use the words of Professor Wilson) "no more isotropic than those of the mollusk or annelid." It seems probable that this is true for other sea-urchin eggs as well.
6. In some cases the two incomplete skeletons of a pair of blastomeres from the same egg are complementary. This suggests further a certain amount of bilateral organization of the skeleton forming material already localized in the egg at the time of the first cleavage. This idea receives support from the observations of Runnström on *Paracentrotus*.

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THE NATURAL GROUPING OF THE BREMIDÆ
(BOMBIDÆ) WITH SPECIAL REFERENCE
TO BIOLOGICAL CHARACTERS.¹

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In the introductory pages of his rather negativistic treatise on the psychobiology of bumblebees, Wagner ('06), after calling attention to the immense taxonomic importance of biological characters in the case of spiders, cites—by way of contrast—several examples of the disconcerting polymorphism in the coloration and habits of bumblebees and comes to the following pessimistic conclusion:² "These are the facts which compel us from the very beginning to give up all hope of finding fixed and unalterable characters in the taxonomy of bumblebees for the elucidation of the biology of these insects, and *vice versa*, of deriving suitable data from their biology for taxonomic purposes."

As we shall see later, this statement of Wagner ('06), as so many other of his assertions (cf. von Buttel-Reepen, '07, '14; Wheeler, '19; and Plath, '23b, '24), is based upon superficial observations and is wholly untenable. During the past fifty years, several important attempts, most of which antedate Wagner's ('06) work, have been made to subdivide the Bremidæ into natural groups. These subdivisions have been based either upon (1) coloration, (2) structure, or (3) habits, and in some cases on two or all three of these.

In this paper the writer wishes to discuss in detail those attempts to subdivide the Bremidæ into natural groups which have been based primarily upon biological characters, and to sub-

¹ Contributions from the Entomological Laboratory of the Bussey Institution, Harvard University, No. 247.

² "Dies sind die Tatsachen, welche uns von allem Anfange an zwingen, jede Hoffnung darauf aufzugeben, in der Systematik der Hummeln feste und unveränderliche Züge für die Aufklärung der Biologie dieser Insekten zu finden und umgekehrt aus dem biologischen Kriterium geeignete Angaben für die Systematik der selben . . . zu schöpfen."

ject these latter to a critical examination in the light of extensive biological observations on some of our American species. Before doing so, it seems desirable however to review briefly several efforts at grouping which have been made on the basis of (1) coloration and (2) structure.

GROUPING ON THE BASIS OF COLORATION.

The Austrian entomologist von Dalla Torre ('82) is apparently the only one who attempted to subdivide the Bremidæ on the basis of coloration. However, since many species of the genus *Bremus* (*Bombus*) which are not closely related show very striking similarities in coloration, *e.g.*, *Bremus americanorum*³ and *Bremus terricola*, this method of grouping is a very artificial one and for this reason has not been favorably received by other workers. In this connection the present writer would like to confess that he himself, not long ago (cf. Plath '22a, pp. 40-41), made a similar mistake with respect to the genus *Psithyrus*.

GROUPING ON THE BASIS OF STRUCTURE.

There have been several attempts made to group the Bremidæ on the basis of structure. The first of these was by the Russian General Radowszowski ('84) who divided the genus *Bremus* into eleven groups. This scheme of grouping was later somewhat modified by Franklin ('12/'13) in his "Bombidae of the New World," in which the then-known, eighty-five American species of the genus *Bremus* are divided into seven groups, a procedure which has been followed by other American workers. This method of grouping has been further extended by Franklin ('12/'13) to the American species of the genus *Psithyrus* which he divides into three groups.

Another scheme of grouping which has much in common with Radowszowski's ('84) is that of Vogt ('11) who divides the genus *Bremus* into nine subgenera, which Ball ('14) and Krüger ('16 and '20) later increased to ten and thirteen respectively. The last-named author, in addition to making a few modifications in

³ Dr. Joseph Bequaert and the writer have recently studied De Geer's description and figure of *Bremus pennsylvanicus*, and, like Dr. T. H. Frison ('23), have come to the conclusion that it is better to use the name *americanorum* until the type specimen of De Geer is located.

the nine subgenera established by Vogt ('11), has divided the genus *Bremus* into two sections, on the basis of the presence or absence of a spinous projection on the posterior, distal angle of the metatarsus of the middle legs. This character may prove of considerable importance in determining the genetic relationship of the various species of bumblebees, since, as we shall see later, it seems to go hand in hand with certain fundamental differences in the method by which these insects feed their larvae.

Another independent subdivision of the non-parasitic bumblebees on the basis of structure is that by Robertson ('03), who removed certain species from the genus *Bremus* (*Bombus*) and erected the genus *Bombias*, chiefly on the basis of the size and position of the ocelli. This innovation does not seem to have found favor with European workers, but *Bombias*, either as genus or subgenus, is in common use in the United States.

Among the attempts to divide the Bremidae into natural groups on the basis of structure, we may also include that of Friese and von Wagner ('10), who constructed a "Stammbaum" which is intended to show the probable genetic relationship of the fifteen species of *Bremus* occurring in Germany. This scheme of grouping will, no doubt, have to be considerably modified, since it is not in harmony with certain fundamental biological and structural characters which have been pointed out by Sladen ('99 and '12) and Krüger ('16 and '20).

GROUPING ON THE BASIS OF HABITS.

The first one who used biological characters for subdividing the Bremidae was the English entomologist Smith ('76). In the second edition of his "Catalogue of British Bees," he divides the English species of the genus *Bremus* into Surface-builders (Section I.) and Underground-builders (Section II.). While this method of subdividing the Bremidae may be of some value to the amateur naturalist, it is of little importance for taxonomic purposes, since certain species of bumblebees, *e.g.*, *Bremus vagans*, make use of many diverse nesting sites: underground, on the surface, in stone walls, hollow trees, birds' nests, attics, fur coats, etc.

A more promising and dependable biological character for

dividing the Bremidæ into natural groups was discovered by the late F. W. L. Sladen ('99) who called attention to the fact that the various English species of the genus *Bremus* employ two very distinct methods of feeding their larvæ. Since this short, but epoch-making paper of Sladen ('99) has been overlooked by several workers, and since it forms the chief basis for the present discussion, it seems advisable to quote the two following paragraphs from it.

"Taking nests of humble-bees and keeping them under observation in specially constructed hives has been a hobby of mine for some years, and it is astonishing what a quantity of interesting information one can gather in this way about the habits and life-history of the dozen or so species that have been recorded from this country, several of which are very common and familiar to every one. It appears that each species has habits and proclivities more or less peculiar to itself, and these, if they could be accurately observed and recorded, would help very much in the systematic arrangement of the species, which in this interesting genus is unusually difficult, owing to the lack of easily recognisable structural differences, and to the little reliance that can be placed on colouring.

"As a result of taking a number of nests it appears that most of the *Bombi* found in this country may be separated into two groups, on what seems to be a rather important difference in the manner of raising their young. These groups may be conveniently named (1) the '*pouch-makers*' and (2) the '*pollen-storers*.' The *pouch-makers* form little pockets or pouches of wax at the side of a wax-covered mass of growing larvæ, into which the workers drop the pellets of pollen direct from their hind tibiæ on the return to the nest from the fields. The *pollen-storers*, on the contrary, store the newly gathered pollen in waxen cells specially made for the purpose, or in old cocoons specially set apart to receive it, from which it is taken and given to the larvæ through the mouths of the nurse-bees as required."

Thirteen years later, Sladen ('12, pp. 40-44, 152-153), in addition to elaborating the foregoing plan in general, changed the term *Pouch-makers* to *Pocket makers*, and further subdivided the latter group into *Pollen-primers* and *Carder-bees*. However,

shortly after this revision had appeared in print, Sladen ('12, pp. 274-275) discovered that the character on the basis of which he had established the *Pollen-primers* is not a dependable one and hence inserted the following paragraph among his "Additional Notes": "*B. latreillellus* was considered to be a pollen-primer because pollen was found under the eggs in a nest in an advanced stage examined in 1911. But in a nest in an earlier stage kept under observation in 1912 the eggs were laid in cells that contained no pollen, although in at least one case pollen was put into the cell and removed before the eggs were laid. Should future investigation show that *latreillellus* is a pollen-primer only under abnormal conditions, a better name for the group, consisting of *ruderatus*, *hortorum*, *latreillellus* and *distinguendus*, would be 'Long-faced Humble-bees'; the term 'pollen-primers' could then be restricted to *ruderatus* and *hortorum*. *Latreillellus* and *distinguendus* are not closely related to *ruderatus* and *hortorum*."

For several summers the present writer has devoted most of his time to the study of the biology of bumblebees, and has had occasion to examine the nests of about 200 *Bremus* colonies belonging to the following North American species: *affinis*, *americanorum*, *bimaculatus*, *fervidus*, *impatiens*, *perplexus*, *occidentalis*, *separatus*, *ternarius*, *terricola*, and *vagans*. This material furnished an excellent opportunity to test the soundness of Sladen's ('99 and '12) classification, as applied to four of our seven American groups. The results of this investigation show that Sladen's ('99 and '12) classification provides an excellent basis for further taxonomic and phylogenetic work, but that certain details of his scheme will have to be modified.

As already stated, Sladen ('12) divides the English Bremidae into two main groups, the *Pollen-storers* and the *Pocket-makers*. In regard to the last-named group Sladen ('12, p. 44) says: "When the usual receptacles for pollen employed by a particular species are not available, it may adopt those employed by others. Thus in a strong nest of *B. agrorum*, one of the pocket-making species that I had under observation in 1910, the workers, during a period when there were no growing larvae and consequently no pockets for pollen, dropped all the pollen they brought home

into a special waxen cell they had constructed, like *terrestris*, on the top of some cocoons. Also a colony of *B. hortorum*, another pocket-maker, being in an advanced stage, and having no growing larvae, placed pollen in the cocoons vacated by the young queens, but only lined the interior of the cocoons with it."

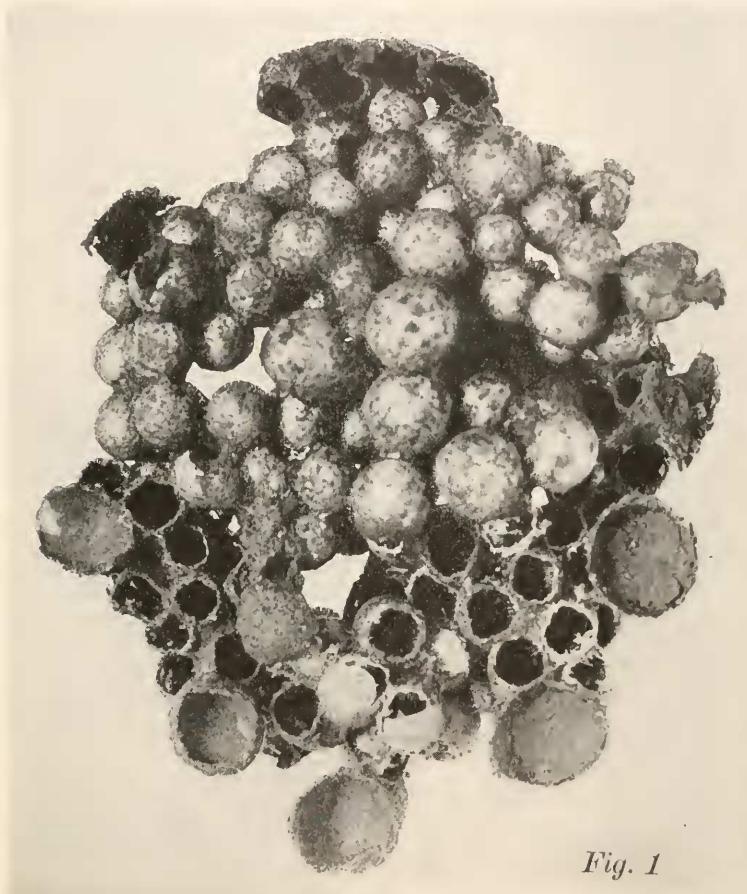


Fig. 1

FIG. 1. Comb of *Bremus fervidus* with five pollen bowls on the lower half of the periphery. Natural size.

In this connection the present writer would like to call attention to the fact that *Bremus americanorum* (cf. Franklin, '12/'13 p. 405) and *Bremus fervidus*, two of our American *Pocket-*

makers, not only store pollen under the conditions specified by Sladen ('12, p. 44), but that toward the end of the summer—at the height of brood-rearing—prosperous colonies of these two species store considerable quantities of pollen, in some cases in shallow waxen bowls which are usually constructed near the periphery of the comb. (cf. Fig. 1). From these facts it is evident that Sladen's ('99 and '12) term *Pollen-storers* is of little use in any comprehensive classification of the Bremidae.

Furthermore, in regard to certain *Pocket-makers*, e.g., *B. americanorum* and *B. fervidus*, it must be pointed out that the habit of making pockets is resorted to only in the case of those larvae which are destined to become workers, while the brood which furnishes the other two castes (queens and males) is fed by regurgitation; i.e., in the same manner as are those of the non-pocket-making species, a fact to which the writer has called attention in an earlier paper (cf. Plath '23a, p. 339). However, since this method of feeding the male and queen larvae is not employed—at least in the temperate regions—until toward the end of the breeding season, and since the method by which these Bremidae feed their worker brood is quite distinctive, it seems best to retain the term *Pocket-makers*, unless future investigations show that the use of this term is impracticable.

We now come to Sladen's ('12) division of the *Pocket-makers* into *Pollen-primers* and *Carder-bees*. As already mentioned, Sladen later ('12, pp. 274-275) removed two species from the *Pollen-primers*; but, as will be seen from the following observations, the term *Pollen-primers* will have to be completely rejected as a subdivision of the *Pocket-makers*.

In the fall of 1921 the present writer (cf. Plath '22a, p. 34 and '22b, p. 195) made detailed observations on a large colony of *Bremus impatiens*, a non-pocket-making species, and in this case the egg-cells were regularly primed with pollen pellets before the workers oviposited in them.⁴

Even more unsatisfactory is the term "Long-faced Humble-bees" which Sladen ('12, p. 275) introduced later, since *Bremus mendax*, which structurally belongs to an entirely different group,

⁴ Incidentally this observation also shows that von Buttel-Reepen's ('03, p. 35) explanation of the pollen priming habit of bumblebees is incorrect (cf. also Sladen, '12, pp. 274-275).

and probably is a non-pocket-making species, has a longer face (cf. Krüger, '20, pp. 310 and 359) than either of the two species mentioned by Sladen ('12, p. 275).

Equally impracticable is the term *Carder-bees* which Sladen ('12, pp. 152-153) applies to the second subdivision of the *Pocket-makers*. The collecting of nesting material, the character on which Sladen ('12, p. 17) based this group, is more or less common to all species of the genus *Bremus*, including the non-pocket-making species. However, by the rejection of the term *Carder-bees*, the writer does not wish to imply that the species which Sladen ('12, p. 152) includes under this term do not constitute a distinct group.

Having pointed out the inadequacy of Sladen's ('12) classification as applied to some of our North American Bremidæ, the writer would suggest the following changes in Sladen's ('12) scheme. The name *Marsipæa*⁵ (from Greek *marsipos*, a pouch, and *poiein*, to make) is proposed as a substitute for Sladen's ('12) term *pocket-makers*. This change would result in a suitable name for Sladen's *pollen-storers* (the non-pocket-making species) which could then be designated as the *Amarsipæa*. For reasons already stated, it is further suggested that the terms *Pollen-primers*, *Long-faced humble-bees*, and *Carder-bees* be dropped as subdivisions of the *Marsipæa*.

Concerning two species belonging to the *Amarsipæa*, Sladen ('12, p. 36) says: "The larvae of *B. terrestris* and *lucorum* do not keep together in a compact mass, but as they begin to grow large each one acquires its own covering of wax, although they do not separate completely; the cocoons, therefore, do not form definite clusters, and are easily detached from one another." And in the next paragraph he continues: "With most of the species the skin of wax that covers each batch of larvae is to the unaided eye unbroken, but as the larvae grow, *B. terrestris*, *lucorum*, and *latreillellus* leave visible holes in the wax, which, when the larvae approach full size, become large. The larvae would now run the risk of falling out of their soft wax covering, which would mean their destruction, for a naked larva is always carried out of the nest; but they avoid this danger by enclosing themselves in a

⁵ The writer here wishes to express his thanks to Professors W. G. Aurelio and W. M. Wheeler for their valuable suggestions concerning these terms.

loose web of silk, doing this a day or two before they begin to spin their cocoons."

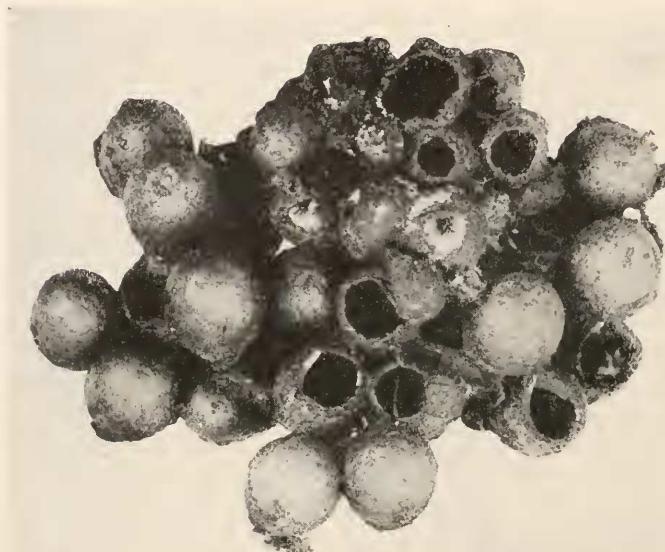


Fig. 2

FIG. 2. Comb of *Bremus affinis* showing exposed larvæ. Natural size.

In this connection the writer would like to call attention to the fact that some of our American *Amarsipæa*, e.g., *Bremus affinis*, *occidentalis*, and *terricola*, have the same habit (cf. Fig. 2). Quite contrary to this treatment of the larvæ is that of the other American and European *Amarsipæa* whose habits have been studied, for the latter carefully keep their larvæ covered with wax (cf. Fig. 3).

Because of this difference in habit, the present writer proposes the name *Phaneroschadonenta* (from Greek *phaneros*, visible, and *schadon*, a bee larva) for those *Amarsipæa* whose larvæ are visible during the greater part of their development, and the name *Cryptoschadonenta* (from Greek *cryptos*, hidden) for those *Amarsipæa* which keep their larvæ covered with wax.

In addition to the very loosely connected cocoons and the uncovered larvæ, the *Phaneroschadonenta* exhibit a number of other peculiarities which mark them as a distinct group. Among

these may be mentioned (1) the short antennæ of the males, (2) the similarity in form between males and workers, and (3) the extreme shortness of the head.⁶ This last character is coupled



Fig. 3

FIG. 3. Comb of *Bremus impatiens*, a typical example of the *Cryptoschadonenta*. Natural size.

with a short tongue, which makes it necessary for this group of bumblebees to perforate long-tubed flowers in order to secure the nectar. Because of this habit they have been given the name *Disteleologists* (cf. Haeckel, '66; Müller, '79; and von Buttelen-Reepen, '14), a subject which the writer intends to discuss in another paper.

The *Phaneroschadonenta* also have the following interesting characteristics: (1) they construct—usually near the center of the comb—one or more bulky, waxen tubes in which they store large quantities of pollen (cf. Figs. 4 and 5); (2) the wax which they produce is unusually dark and brittle, as if mixed with black soil; and (3) their males, like those of the genus *Psithyrus*, are rather sluggish as compared with those of other species.⁷ The writer would here also like to point out that the *Phaneroschadonenta* appear early in the spring, that they do not nest on

⁶ Cf. Radowszowski ('84), Friese and von Wagner ('10), Vogt ('11), Franklin ('12/'13), and Krüger ('20).

⁷ Cf. Schmiedeknecht ('78), Hoffer ('82, '82/'83), Saunders ('09), Sladen ('12), and Frison ('17).

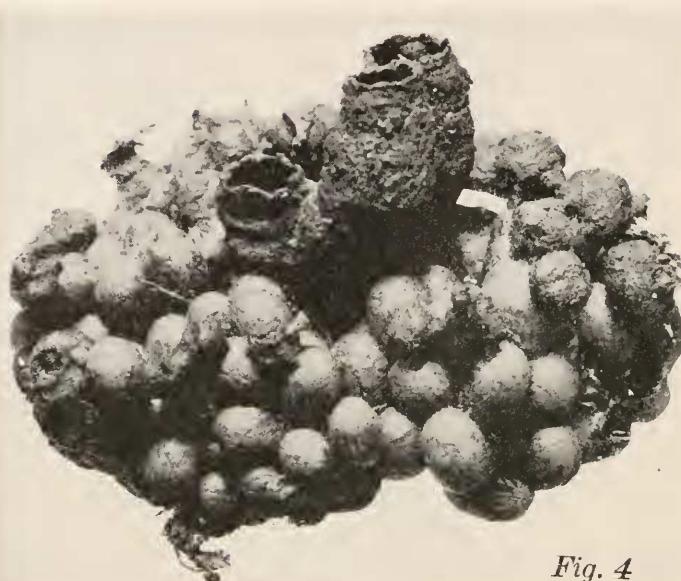


Fig. 4

FIG. 4. Comb of *Bremus affinis* showing three pollen cylinders. Natural size

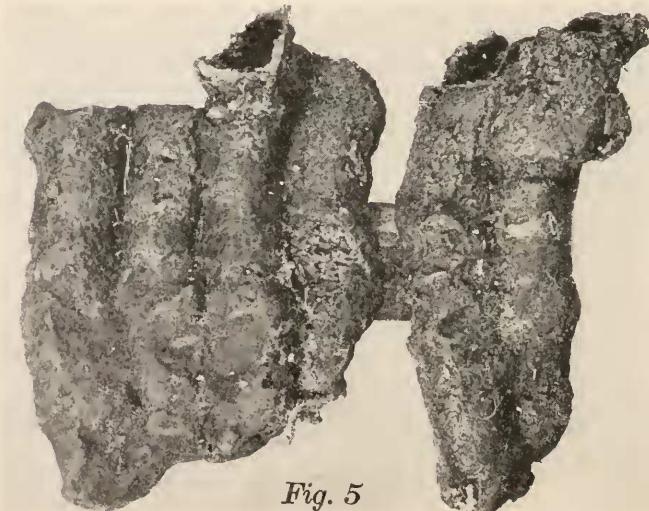


Fig. 5

FIG. 5. Six pollen cylinders taken from a nest of *Bremus affinis*. Natural size
Sometimes more than a dozen of these pollen cylinders are found in one nest.

the surface of the ground, and that—as far as known—they do not occur in South America.

However, it may be necessary to use the terms *Phanero-* and *Cryptoschadonenta* only provisionally, since Sladen ('12, pp. 36-37 and 185) states that *Bremus latreillellus*, a *pocket-maker* (*Marsipæa*), also leaves its larvae uncovered. This seems strange, since the other *Marsipæa* studied are very solicitous to keep their larvae completely covered with wax (cf. Fig. 6), and this fact



Fig. 6

FIG. 6. Comb of *Bremus americanorum* showing (a) three pockets, and (b) worker larvae completely enclosed by wax. Natural size.

suggests the possibility that Sladen's ('12) observations on *Bremus latreillellus* may have been made during extremely hot weather, when, due to the softening of the wax, all bumblebee larvae are likely to become exposed. Should subsequent observations confirm Sladen's ('12, pp. 37 and 185) statement concerning *Bremus latreillellus*, other terms will have to be substituted for *Phanero-* and *Cryptoschadonenta* to bring out the distinctness of this group of bumblebees.

The various American and European species of bumblebees whose methods of rearing their young have been studied up to

the present are listed in Table I. with the suggested modifications of Sladen's ('99 and '12) scheme.

TABLE I.

AMARSIPÆA.

Phaneroschadonenta.

1. <i>Bremus affinis</i>	}	American Species.
2. " <i>occidentalis</i>		
3. " <i>terricola</i>		
4. " <i>lucorum</i> ⁸	}	European Species
5. " <i>terrestris</i> ⁸		

Cryptoschadoneta.

6. <i>Bremus bimaculatus</i>	}	American Species.
7. " <i>impatiens</i>		
8. " <i>perplexus</i>		
9. " <i>ternarius</i>		
10. " <i>vagans</i>		
11. " <i>auricomus</i> ⁹		
12. " <i>separatus</i>		
13. " <i>jonellus</i> ⁸	}	European Species.
14. " <i>lapidarius</i> ⁸		
15. " <i>lapponicus</i> ⁸		
16. " <i>pratorum</i> ⁸		

MARSIPÆA.

17. <i>Bremus americanorum</i>	}	American Species.
18. " <i>servidus</i>		
19. " <i>hortorum</i> ⁸	}	European Species.
20. " <i>ruderatus</i> ⁸		
21. " <i>distinguendus</i> ⁸		
22. " <i>latreillellus</i> ⁸		
23. " <i>agrorum</i> ⁸		
24. " <i>derhamellus</i> ⁸		
25. " <i>helferanus</i> ⁸		
26. " <i>muscorum</i> ⁸		
27. " <i>sylvarum</i> ⁸		

Concerning the relative temper of the *Marsi-* and *Amarsipæa*, Sladen ('99, pp. 230-231) says: "It will be noticed that the *pouch-makers* include roughly the timid species. The *pollen-storers*, on the contrary, consist of the bolder species." This is not true of our New England *Marsipæa*, e.g., *Bremus americanorum* and *Bremus servidus*, these being the most vicious

⁸ According to Sladen ('12, pp. 152-153).

⁹ According to Frison ('17, pp. 284).

bumblebee species with which the present writer has come in contact, while most of our *Amarsipæa* are comparatively gentle.

It will be interesting to see whether the various bumblebee groups which have been established on the basis of structure are really homogeneous species, when their habits are studied from a taxonomic viewpoint. In the light of our present knowledge, we should expect the seven American groups (cf. Franklin, '12/'13) of the genus *Bremus* to belong to the biological subdivisions as indicated in Table II.

TABLE II.

AMARSIPÆA.

*Phaneroschadonenta.*1. *Terrestris* group.*Cryptoschadonenta.*2. *Auricomus* group.¹⁰3. *Fraternus* group.¹⁰4. *Kirbyellus* group.¹¹5. *Pratorum* group.

MARSIPÆA.

6. *Borealis* group.¹¹7. *Dumoucheli* group.

If Krüger's ('16 and '20) subdivision of the Bremidæ into two sections is really as far-reaching as it appears to be, we should expect the members of the *Borealis* group to belong to the *Marsipæa*, and those of the *Kirbyellus* group to the *Amarsipæa*, since the spine is present in the former, but absent in the latter.

It will also be interesting to investigate the methods which the Bremidæ employ in feeding the larvæ of the genus *Psithyrus*, since certain species of the latter, e.g., *Psithyrus variabilis*, breed in the nests of the *Marsipæa*, while others, e.g., *Psithyrus ashtonii*, are parasitic on the *Amarsipæa*.

Due to a lack of sufficient material, the writer has been unable to determine whether the species for which Robertson ('03) erected the genus *Bombias* have any clearly defined biological peculiarities¹² which would serve as suitable biological characters for distinguishing this group.

¹⁰ Belonging to the genus *Bombias* of Robertson ('03).

¹¹ Probably.

¹² The males of this group, like those of closely related European species, have their own peculiar habits (cf. Schmiedeknecht, '78; Hoffer, '82/'83; Robertson,

SUMMARY.

1. Contrary to the assertions of Wagner ('06), biological characters are of considerable importance in determining the relationships between the various species of bumblebees, while structural characters frequently make it possible to draw inferences as to the habits of these insects.
2. Our North American *Marsipaea*—at least *Bremus americanorum* and *Bremus fervidus*—feed only their worker brood through pockets, while those larvae which are destined to become queens and males are fed by regurgitation.
3. The use of the terms *Pollen-primers*, *Carder-bees*, and *Long-faced humble-bees* (cf. Sladen '12) is impracticable in any comprehensive classification of the Bremidae.
4. Von Buttel-Reepen's ('03) explanation of the pollen-priming habit of bumblebees is not substantiated by the biology of some of our North American species.

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BIOLOGICAL BULLETIN

OBSERVATIONS ON THE LIFE-HISTORY OF *AMOEBA PROTEUS*.¹

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There is still considerable diversity of opinion concerning the life-history of *Amœba proteus*. Some hold that it reproduces exclusively by binary fission; others do not agree with this.

Carter ('56) asserts that he observed the nucleus in a given specimen, break up into several small nuclei each of which associated itself with a bit of cytoplasm; and that he then saw the membrane of the amœba break and the small nuclei with their surrounding cytoplasm escape into the external medium and move away by amœboid movement.

Wallich ('63), Scheel ('99), Calkins ('05 and '07), Metcalf ('10), Hausman ('20), and Taylor ('24) maintain that they made similar observations. Scheel and Calkins contend that the amœbæ encyst before the nucleus breaks up and Calkins holds that there is a multinucleated generation which ends by sexual activity. Calkins says, "The fertilized cell of *Amœba* (unknown at present) gives rise to a young amœboid organism formerly known as *Amœba proteus*." Later ('07) he sectioned the amœbæ on which these observations were made and maintains that he found a process of internal fertilization very similar to endomyxis.

Metcalf ('10) asserts that there are two methods of reproduction in which fragmentation occurs; one in which the parent amœba breaks up liberating minute amœboid forms which develop into large amœbæ; another in which small amœboid forms are liberated from the parent by a gemmule formation. These

¹ These observations were made while the authors were working under Dr. S. O. Mast, to whom they are very grateful for timely criticism and helpful suggestions.

amoeboid forms he asserts develop flagella, then fuse in pairs, after which the zygotes thus formed develop into large amoebæ.

Taylor gives a detailed description of the nuclear processes accompanying fragmentation. She maintains that the small fragments as they emerge from the parent amoeba are cysts which hatch out after varying lengths of time.

All of these observations indicate that *Amœba proteus* at times fragments, forming numerous small amoeboid forms, and they appear to indicate that these small forms develop into the large forms which are usually studied. Hausman contends that he actually observed the transformation. There are, however, some prominent investigators, who hold that the evidence presented is not conclusive; for example, Schaeffer, ('26, p. 111.) who says, "We find no proof that the life cycle of the common large ameba includes more than . . . reproduction by fission." The following observations have a definite bearing on this question:

During the last two years we have had under close observation numerous cultures of *Amœba proteus*, started from a few specimens collected in the summer of 1924. During this time it was repeatedly noticed in various cultures that the amoebæ multiplied very rapidly for a while, then suddenly practically disappeared. At first these cultures were all discarded, except a few which were saved for the other protozoa living in them. These were set aside and observed from time to time. After about a week, large numbers of minute amoebæ were found in some of them, and several weeks later numerous large amoebæ.

In further observations it was discovered in several cultures of large amoebæ, (1) that after a period of rapid multiplication by fission the specimens became increasingly more sluggish, darker and more granular in appearance; (2) that they began to decrease in number; and (3) that as the large amoebæ decreased small ones appeared and increased. This did not occur in all cultures, for in some all amoebæ died.

This disappearance of the large amoebæ was observed to occur in several cultures as the solution changed from acid to alkaline in reaction (pH 6.8 to 7.4) or *vice versa*. In other cultures, however, there was no such change, in these the solution remained almost constant as to bacterial content, clearness, amount of food and hydrogen-ion concentration.

Similar results were obtained in cultures on hollow ground slides. All of these cultures consisted of a few drops of filtered culture fluid. One large amoeba was put into each of about half of them, and none in the rest. Numerous small amoeboid forms appeared in many of the former but in none of the latter. A description of a typical experiment follows:

A large sluggish individual was selected, washed three times in about 5 cc. of distilled water and placed on a hollow ground slide in culture fluid which had been passed through number 50 filter paper. This solution contained no amoebae that could be seen under a highpower dissecting binocular, or a 1.9 mm. water-immersion objective. The amoeba was observed from time to time. The second day after it had been put into the culture fluid on the slide, it became extremely sluggish and remained so. The following morning it had disappeared and in the region where it had been, there were from 150 to 200 minute amoeboid forms, about $10\ \mu$ in length. A drop of fresh sterile hay infusion was now added every other day for about a week and it could clearly be seen that the small amoeboid forms were becoming distinctly larger. Unfortunately, at the end of this time too much solution was added and the forms died.

Following these experiments several amoebae were isolated and closely watched for a long period of time. The process of fragmentation was actually observed to occur in a number of cases.

The results obtained seem to prove conclusively that *Amoeba proteus* at times breaks up into small amoeboid forms but they do not prove that these small forms develop into large ones. The evidence presented in the following paragraphs appears, however, to prove this.

On February 19, 1925, two grams of timothy hay were added to 1000 cc. of spring water and boiled for ten minutes. While still hot some of the fluid was poured into a sterile 100 cc. pyrex flask. This was then plugged tightly with cotton. When the flask had cooled a few drops of old culture fluid, which had been passed through number 50 filter paper, were added. The flask was then again plugged with cotton and allowed to stand for a week. Then this culture, free from amoebae as shown by careful observation, was inoculated by one amoeba which had been washed in several

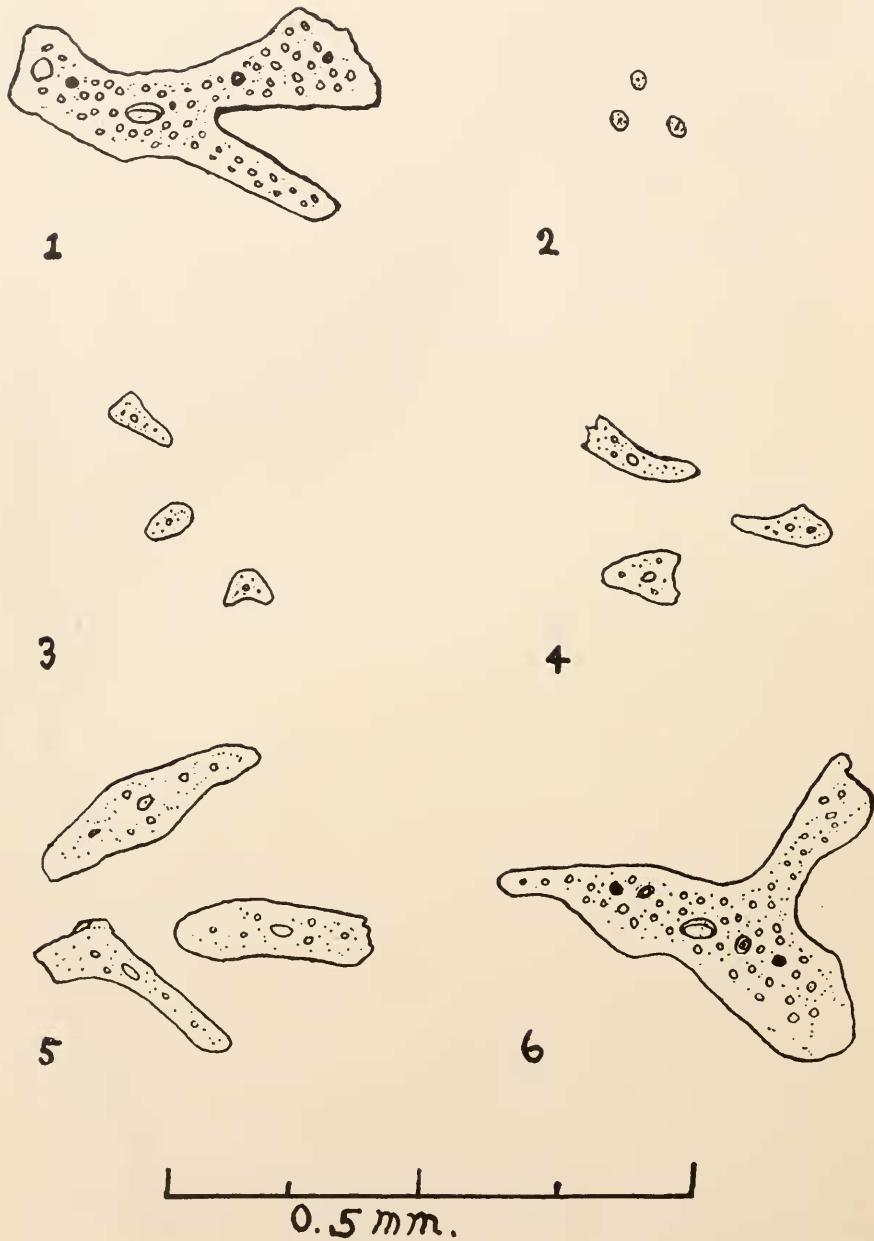


FIG. 1. Camera lucida sketches of some of the largest amoebae in a culture at different time intervals, showing growth of small amoebae into large amoebae; 1, one of the original amoebae which fragmented, giving rise to small amoebae; 2,

changes of distilled water. After this there was added each day for food about 5 cc. of fresh sterile culture fluid like the original culture fluid. All pipettes used in handling the amoeba and the culture fluid were repeatedly sterilized in order to eliminate contamination.

The culture was poured into a sterile shallow dish and thoroughly examined every few days. It was found that the individual multiplied by fission until in about two weeks there were perhaps fifty large amoebae in the culture. Then fission appeared to cease and the amoebae assumed a dark granular color, after which they began rapidly to disappear so that by the end of the third week there were no large amoebae present. If there had been one large amoeba left it could not have escaped the rigid inspection to which the culture was subjected. The culture was now set aside for a week, after which it was again examined. It now contained numerous small amoebae the length of which varied from 10 to 40 μ , but no large amoebae. For over a month following this the culture was repeatedly closely observed. The amoebae increased in size until by the last of April there were numerous specimens which measured 300-600 μ in length.

On September 17, 1926, fourteen cultures made up in a modified Ringer's solution were inoculated with amoebae from an ordinary spring water culture after being washed in six changes of the Ringer's solution. At the end of four days all of the large amoebae had disappeared and soon after this small amoeboid forms were observed in great numbers. From this time on the cultures were thoroughly examined at the end of each week and after each examination camera lucida sketches were made of the largest amoebae found in each culture. Some of these sketches are herewith reproduced (Fig. 1). They show that the amoeboid forms increased from an approximate average volume of 400 cubic micra to one of 250,000 cubic micra and that this increase required two months. During this time, however, it was observed that these small amoebae occasionally divided (Fig. 2). All of

small amoebae produced by fragmentation of original large amoebae; 3, largest amoebae in culture ten days after fragmentation; 4, largest amoebae in culture four weeks after fragmentation; 5, largest amoebae in culture six weeks after fragmentation; 6, largest amoebae in culture eight weeks after fragmentation.

these cultures and the one mentioned in the preceding paragraph, were thoroughly examined at least twenty-five times during the two months growth period, and no large amœbæ could be dis-

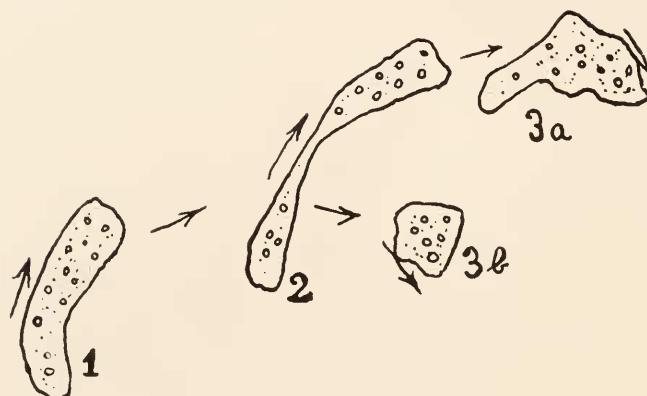


FIG. 2. Camera lucida drawings of a small amœba just before division (1) and (2) and immediately after division (3a) and (3b).

covered until the end of two months as stated. It is, therefore, evident that even one large amœba could not have remained in the large form unobserved during the growth period of nearly two months.

It is evident that the small forms develop into large ones and that as they do so they divide from time to time.

SUMMARY.

Individuals of *Amœba proteus* sometimes break up into from 100 to 300 amoeboid forms. These amoeboid forms gradually become larger until at the end of about two months they are as large as the original specimens. During the increase in size division occurs from time to time.

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AN ANALYSIS OF THE SPAWNING HABITS AND
SPA WNING STIMULI OF *CUMINGIA*
TELLINOIDES.¹

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Since the discovery of a slight lunar periodicity in the spawning of *Chatopleura apiculata*, the writer has studied the spawning seasons of a considerable number of animals in the hope of finding other more clearly marked cases. The conclusion of this study is that the phenomenon is of rare occurrence at Woods Hole. *Cumingia tellinoides* is the one species studied critically to date that shows a lunar periodicity. Three other species whose study has been completed show absolutely no periodicity. Others being studied require further investigation before they can be passed upon with any degree of certainty. If semi-scientific reports are trustworthy, this type of periodicity is much more common in the tropics, since several fairly well authenticated cases are reported from the Tortugas and elsewhere.

A part of the data presented in this paper was read before the American Society of Zoologists at their annual meeting in December, 1922. Since that time the study has been continued and the results are finally presented for publication in their completed form.

SECTION I.

THE SPAWNING SEASON.

The Duration and Characteristics of the Spawning Season.

Cumingia begins to spawn about the middle of June and continues with variations of activity until the middle of September and sometimes until the first of October. During 1926 the first eggs were obtained on June 21 and the last ones on September 21. The heaviest spawning occurs during the last

¹ From the Osborn Zoölogical Laboratory, Yale University, New Haven, Conn., and the Marine Biological Laboratory, Woods Hole, Mass.

week in June, July and the first half of August, as stated by Morgan (*Jour. Exp. Zoöl.*, 1910). The production of eggs by each female is continuous and covers the entire period. If *Cumingia* which have spawned vigorously in the laboratory are returned to their normal habitat for three weeks or a month, they will again set free large quantities of eggs or sperm showing that the supply is replenished.

Experiment No. 1.—July 7, 1924. A stake was driven off Ram Island and eighteen *Cumingia* were planted at its base. All these had just spawned in abundance. On August 7, seven of these *Cumingia* were again brought to the laboratory and treated as usual to induce spawning. After forty minutes two females had spawned heavily and three males had shed sperm abundantly. One other female spawned after one and one half hours. One of the seven did not spawn. The eggs of all these were fertilized and grew into normal veligers.

Experiment No. 2.—July 6, 1926. Eighteen *Cumingia* that had just spawned heavily were planted off Ram Island. On August 1, nine of these were again brought to the laboratory. Three females spawned actively, three males shed sperm in quantity, three did not spawn.

These two experiments indicate that *Cumingia* spawns more than once in a season and that the production of eggs and sperm is continuous.

There are indications that each female spawns two or three times during the season when left in the natural habitat. This inference is founded upon the facts that the eggs are constantly produced and that spawning seems to be most common at full moon and the days following full moon. Since there is no direct evidence on this point, I do not care to insist upon it, except to refer to the schedule of spawning experiments. (See tables, Section III. on lunar periodicity.) There are clearly marked variations in spawning activity which appear to be associated with phases of the lunar cycle.

Orton 15 has advocated the theory that the duration of the spawning seasons of animals is determined by temperature. According to his conception, spawning by any summer breeding species begins in the spring when the water reaches a certain

temperature and ends in the fall when the water drops below that temperature. It may be in order to say that this theory is true only in a very general sense, since the species that have been studied by me do not agree among themselves in this regard. Thus *Cumingia* began to spawn on June 21, 1926, when the temperature of the water was 60° F. and quit about September 25 when the temperature was 65° F. *Bugula flabellata* *at* began spawning on June 20 in 1926 when the temperature was 60° F. and continued until November 26, when the temperature was 47° F. *Bugula* usually begins to shed its larvæ by June 10 and continues until late in November, which makes the discrepancy in temperature less pronounced, June 10 temperature being approximately 55° F., November 25 temperature 45° F. *Chætopleura* begins to spawn about June 25, temperature 60° to 65° F. and ends early in September, temperature 65° to 70° F.

Spawning is caused at particular times in nature by various specific stimuli and it is not determined by temperature alone.

Orton gives convincing evidence to show that temperature is the chief factor in determining the limits of the spawning seasons of marine invertebrates. He, however, dismisses the factor light with too little emphasis. Although most tropical animals may spawn throughout the long summer in accordance with his theory, the Palolo worm spawns on one or two days only; and the ripening of its gametes is influenced by light according to several investigators. Light plays a part both in the maturing of the gametes and in spawning in several well-known cases. A very striking example is *Dictyota* *at* Beaufort, N. C., as described by Hoyt. Temperature, no doubt, is the chief factor in determining the spawning season just as it is also the most important factor in growth and all protoplasmic activity.

SECTION II.

ARTIFICIAL SPAWNING STIMULI.

I. Shock.

As stated in my paper on *Chætopleura* (BIOL. BULL., 1922), certain marine invertebrate animals will spawn when placed in an artificial or unusual environment, stimulated apparently by

shock. As a case in point, if the tube forming annelid *Hydroides* is removed from its shell and the exposed worm placed in a dish of sea water it will spawn at once, the eggs or sperm seeming to come from every nephridium of the body. This worm will always spawn under these circumstances whether the gametes are fully mature or not. Half-grown eggs are as readily spawned as mature ones. *Hydroides* will spawn in the spring before any of the eggs are even approximately mature.

In like manner, *Cumingia* spawn readily in the laboratory. It is only necessary to place them in a bowl of sea water and allow them to remain undisturbed for half an hour or forty-five minutes. They usually begin to spawn in half an hour. The gametes are expelled through the dorsal siphon which is extended to a great length. As the eggs or sperm accumulate in the siphon they are thrown out forcibly by sudden whip-like contractions of these organs. During the height of the breeding season it frequently happens that every individual brought to the laboratory spawns. It is not to be supposed that all would have spawned on that particular day if left in their natural environment. There is then something about the treatment which involves digging them from the sand and subjecting them to various unusual disturbances which stimulates spawning. It is safe to conclude that this spawning stimulus is shock as in the case of *Hydroides*. Spawning takes place just the same when sea water is allowed to flow gently through the dish so that the accumulation of CO_2 is not a factor. It is to be noted that shock is an abnormal stimulus. It may be said again that laboratory experiments are frequently unreliable guides to natural behavior and may lead to misinterpretation if depended upon too implicitly.

CHEMICAL STIMULI.

The question next arises: do the sexes stimulate each other in any way? The question as to whether the females are stimulated to spawn by the presence of the male and vice versa has frequently been discussed in connection with various species. Some very clear cases of the existence of such a chemical stimulus have been discovered. This, for instance, is the case in *Nereis limbata* as described by Lillie and Just. When placed together in a dish

of sea water both sexes are excited at once to remarkable activity, and while swimming excitedly they expel their sexual products with violent contractions. Galtsoff has recently shown that the female oyster will spawn if living oyster sperm is added to the water. This is opposed to the published statements by Nelson who has thought that the oyster spawns on particularly warm days and that general spawning on oyster beds is induced by temperature. I am under the impression that he is correct in this statement but the implied conclusion that temperature is the only spawning stimulus may be misleading. He shows, however, that spawning occurs on the peaks of rising temperatures which indicates that changes in temperature upward cause males to shed and hence general spawning by all nearby oysters results. Spawning by oysters may take place at any temperature between 68° and 85° F. (Churchill and Nelson).

It was found in the case of *Cumingia* that there is no perceptible stimulus from the opposite sex. They appear to spawn quite as readily when isolated as when in the same dish. Experience has shown that the best way to obtain clean eggs in convenient form for study is to wash the animals free from debris and isolate them in small slender dishes half filled with sea water, or enough water to cover the animal. Both males and females will shed their gametes when so isolated. The eggs are thus obtained free from sperm and may be artificially fertilized at will. Drew was the first to use this method and he was under the impression that drying accentuates or constitutes the spawning stimulus. Morgan in 1910 noted that *Cumingia* will spawn when isolated (*Jour. Exp. Zoöl.*, Vol. 9, p. 595). He thinks, however, that the presence of spermatozoa in the water may incite the females to spawn more promptly than they otherwise would. The stimulus in this case, if authentic, might be either chemical or physical. One is likely to gain the same impression by watching the spawning of *Chatopleura* (*Chiton*) because when males and females are placed together in a dish of sea water, the males always shed their products first, and are followed promptly by the females. So far as could be learned, however, spawning by the females occurred as promptly without spermatozoa as with them.

It is the writer's belief that the spermatozoa have no effect. The mechanical shock of removing the animals from their normal situations furnishes all the stimulus that is required to induce spawning. There is no perceptible stimulus from the opposite sex either chemical or physical. This is shown by a series of experiments designed to test the theory. The three described herewith are representative. The method was to collect *Cumingia* in large numbers. Half of them were isolated in small stender dishes and covered with sea water in the usual way. The other half were treated in the same way except that they were all put in a large crystallization dish so that they might receive chemical stimuli from each other if such exist. The time of spawning is shown in the two columns of the tables and they may be readily compared.

Experiment No. I.

August 21. Collected 20 *Cumingia*. Ten were isolated in stender dishes and ten were placed in a common dish. Experiment set at 12:30 P.M.

10 Isolated Individuals Spawned as Follows.	Time Elapsed before Spawning.	10 in Common Dish Spawned as Follows.
1:15 P.M. one female.....	45 minutes	
1:16 P.M. one male.....	46 "	
1:20 P.M. one male.....	50 "	
1:23 P.M. one male.....	53 "	
	55 "	One male shed 1:25 P.M.
	57 "	One male " 1:27 P.M.
1:29 P.M. one female.....	59 "	
	60 "	One male " 1:30 P.M.
	61 "	One female " 1:31 P.M.
	67 "	One male " 1:37 P.M.
	68 "	One male " 1:38 P.M.
	70 "	One male " 1:40 P.M.
	71 "	One female " 1:41 P.M.
1:55 P.M. one female.....	85 "	
2:05 P.M. one female.....	95 "	

Comment: Three of the isolated individuals and two in the common dish failed to spawn. Comparison shows that those in the common dish did not spawn more promptly than the isolated individuals. It is noteworthy that those in the common dish

spawned close together in point of time. This was also true in Experiment No. 3, but not in No. 2.

Experiment No. 2.

August 27. Collected 34 *Cumingia*. Isolated seventeen in stender dishes and placed seventeen in a common dish. Experiment set at 5:55 P.M.

17 Isolated Individuals Spawned as Follows.	Time Elapsed before Spawning. 30 minutes	17 in Common Dish Spawned as Follows. One female at 6:25 P.M.
One male at 6:27 P.M.	32 "	
One male at 6:28 P.M.	33 "	
One male at 6:29 P.M.	34 "	
	35 "	Two males at 6:30 P.M.
	36 "	One male at 6:31 P.M.
One female at 6:32 P.M.	37 "	
	38 "	One male at 6:33 P.M.
	39 "	One male at 6:34 P.M.
	41 "	One female at 6:36 P.M.
One male at 6:37 P.M.	42 "	One male at 6:37 P.M.
One female at 6:37 P.M.	42 "	
One male at 6:39 P.M.	44 "	
	47 "	One male at 6:42 P.M.
	48 "	One male at 6:43 P.M.
One male at 6:44 P.M.	49 "	
One female at 6:45 P.M.	50 "	
Two males at 6:46 P.M.	51 "	One female at 6:46 P.M.
	52 "	One female at 6:47 P.M.
	55 "	One male at 6:50 P.M.
One female at 6:51 P.M.	56 "	
One female at 6:54 P.M.	59 "	

Comment: Five isolated individuals did not spawn within the hour and five of those in the common dish did not spawn. There is no evidence that they receive a chemical stimulus from each other calculated to induce spawning. They spawn as readily when isolated as when in the same water.

Experiment No. 3.

August 28. Collected 24 *Cumingia*. Isolated twelve in stender dishes and placed twelve in a common dish. Experiment set at 1:30 P.M.

12 Isolated Individuals Spawned as Follows.	Time Elapsed before Spawning. 2 minutes	12 Individuals in Common Dish Spawned as Follows.
1:48 P.M. one male.....	18 "	One male at 1:32 P.M.
	25 "	One male at 1:55 P.M.
	27 "	One male at 1:57 P.M.
1:58 P.M. one male.....	28 "	One male at 1:58 P.M.
1:59 P.M. one male.....	29 "	One male at 1:59 P.M.
	30 "	One female at 2:00 P.M.
	35 "	One male at 2:05 P.M.
	38 "	One male at 2:08 P.M.
	39 "	One male at 2:09 P.M.
2:10 P.M. one female.....	40 "	
2:13 P.M. one female.....	43 "	
2:15 P.M. one male.....	45 "	
2:16 P.M. one male.....	46 "	
2:17 P.M. one female.....	47 "	
2:20 P.M. one female.....	50 "	
2:22 P.M. one female.....	52 "	

Comment: Two of the isolated individuals and three in the common dish did not spawn within an hour. As a matter of chance most of the females got into the isolated dishes.

SECTION III.

NATURAL SPAWNING STIMULI.

I. Lunar Periodicity.

If *Cumingia* spawns most frequently at the period of the full moon, it is evident that there is some cosmic stimulus which varies with the moon's phases to which spawning is due. The two chief variable quantities are light, and tides, or pressure. There is at present no scientific explanation of lunar periodicity although numerous cases are known.

It has been the writer's chief interest during the past five years to learn whether the phenomenon is rare or of common occurrence among animals at Woods Hole. The data bearing on this subject are given in a short forthcoming paper, but the findings in reference to *Cumingia* are given here in greater detail.

A careful study and comparison of data collected during the past five years shows that there is a lunar periodicity in spawning, although it is not as well marked as in *Nereis limbata* or the Suez sea urchin studied by Fox.

There is no clock-like regularity in the spawning of *Cumingia* in nature. Not all individuals spawn promptly at the full moon. Spawning in fact covers a period of two or three weeks in each month. We know this by the quantities of eggs spawned in the laboratory by any particular lot of sexually mature adults brought in. Some set free a maximum quantity, some a small quantity and some only a few eggs or none at all. The last are considered to have spawned recently. By this indirect reasoning, one learns that there is no one particular day on which general spawning occurs. It is likely that spawning occurs when the gonads and their ducts become filled to capacity and this internal pressure no doubt constitutes a second natural spawning stimulus. If the production of gametes were continuous, and uniform in rate, spawning might occur on any day in the month except for this cosmic stimulus which brings about a more or less marked rhythm. I am of the opinion that there is a rhythm in the rate of production of the gametes as well as in the spawning of them. In any case, the only time in the month during which most of the gonads seem empty and during which spawning stimuli seem to be suppressed is the period of the first quarter. Beginning at full moon the heavy spawning is sometimes completed before new moon but more frequently not until near first quarter. In many respects *Cumingia* resembles *Toxopneustes*, the Beaufort sea urchin, whose periodicity was described by Tennent. The production of gametes in this species is rapid and the gonads are soon replenished after spawning occurs.

It should be noted here that there is a distinction between stimuli which bring about the act of spawning and those which bring about the maturing of the gametes. The latter may be an evenly continuous process or it may be enhanced at certain times. The former is merely the act of extruding these gametes and may be induced by some external stimulus, such as those due to the moon in its various phases, or to any other external stimulus.

In the following paragraphs I give the history of this study of *Cumingia* as constituting an important part of the experimental basis for the conclusions drawn.

During the greater part of the breeding season of *Cumingia*,

no lunar periodicity in spawning is likely to be observed unless attention is directed to it. *Cumingia* has been used as a source of embryological material for class work for many years and only rarely has difficulty been experienced in obtaining eggs in abundance at any time that they have been needed. It appeared that eggs could be obtained at any time during the summer. It was only after receiving a suggestion from Heilbrunn that any convincing evidence of periodic spawning was obtained. He said that *Cumingia* would spawn a second time after its season had apparently ended in August. Morgan gives the spawning season of *Cumingia* as June, July, and August, so that he evidently overlooked the September spawning after the break in August.

It was at Heilbrunn's suggestion that I undertook experiments late in the summer of 1922, when, as he said, a break could be expected.

During the week of August 23 to September 1, 1922, no eggs could be obtained from *Cumingia*, although the characteristic spawning reactions were carried out as usual. The siphons were extended to great length and the whip-like lashing of these organs was carried out. All that was expelled, however, was a considerable amount of mucus containing at best a few immature or defective eggs. There were no mature eggs in the ovaries.

Bean, working in the same laboratory, was likewise unable to obtain eggs during this period for experimental purposes. Bean worked constantly with *Cumingia* during the summer of 1922 and he thought that he detected a periodicity during the height of the breeding season. The maximum spawning periods, according to his statement, occur at new and full moon, being therefore bimonthly. This is interesting, if correct, as indicating that the spawning stimulus might be associated with the tides rather than with moonlight. Further study during the summers of 1923, 1924, 1925 and 1926 shows conclusively that the maximum spawning comes at full moon and is not bimonthly.

The table for 1922 gives the exact data as obtained by the writer. It will be seen that spawning activity is revived somewhat before full moon after a period of complete cessation. Furthermore a careful study of the tables for the years 1922, 1924 and 1926 shows that the heaviest spawning occurs at the

period of the full moon until new moon, and that the period of the first quarter is the period of restricted spawning. This behavior of *Cumingia* can scarcely be explained on any other ground than as a lunar effect.

There is no adequate way of describing the variations in spawning that are so obvious to the experimenter. However, the tables of data appended to this section, together with the comments written at the time the experiments were performed, must suffice to explain the basis of the conclusions arrived at. Although they are long, there seems to be no way to give the evidence more briefly. I give the data for three years only as representative.

DISCUSSION.

There has been much speculation about the cause of lunar periodicity in spawning, but it has for the most part remained speculation and guessing. It can never be solved except by experiments similar to those devised by Mayer. Neither *Cumingia* nor *Chatopleura* is suitable for a study of the causes which have produced lunar periodicity. In the first place, periodicity is not clear-cut in these species and in the second place they can be observed only under laboratory conditions which have been shown to be unreliable. They are affected by shock whereas some species are apparently not so affected.

A casual survey of known cases of lunar periodicity shows a general similarity in all and it is likely that the underlying causes are the same in all. A study which undertakes to explain these phenomena should include a search for other cases and especially all worms that are known to show a lunar periodicity should be studied in detail for purposes of comparison. However, speculation and comparison can scarcely explain it. There is need for experimental methods and the Palolo worm, and the Suez sea urchin or possibly *Nereis limbata* are among the most favorable species for study. It should be a comparatively simple problem to subject them to artificial light, especially polarized light, in addition to all the light to which they are accustomed, to see if they can be thrown out of tune. The Palolo worm seems to offer the best opportunity because of its definite and predictable

time of spawning, so that any change from this time could be regarded as an experimental modification.

SUMMARY.

1. The spawning season of *Cumingia* extends from the middle of June until the middle or end of September. The most active spawning usually includes the last week in June and ends about August 20. Each female spawns more than once and the production of gametes is practically continuous.
2. The spawning is heaviest from full moon to new moon and least at first quarter thus showing a lunar periodicity.
3. *Cumingia* is greatly affected by shock and rough treatment so that sexually mature individuals spawn promptly when brought to the laboratory, after being placed in a bowl of quiet sea water.
4. There are apparently no chemical stimuli by which the sexes stimulate each other to sexual activity as is the case in *Nereis limbata* and the oyster. They spawn as readily when isolated as when in close proximity.
5. It is shown that temperature is not the only factor which determines the duration of the spawning season and periods of spawning.

SPAWNING EXPERIMENTS. 1922.

CUMINGIA TELLINOIDES.

Table No. I.

Cumingia spawned vigorously during the first half of August but stopped suddenly about August 15. Several investigators were experimenting upon *Cumingia* eggs at that time and noted this fact. All collections were made at low tide or within an hour of low tide at the convenience of the supply department of the laboratory.

* September 21, new moon.

I. 8/23. No eggs spawned. 1 male shed sperm abundantly. 17 did not spawn.

II. 8/24. No eggs and no sperm.

III. 8/27. No eggs spawned. 4 males shed sperm small amount. 14 did not spawn.

* September 28, first quarter.

IV. 9/1. 5 females spawned in quantity. 6 males shed sperm, 3 in quantity, 3 in small amount. 7 did not spawn.

Comment.—This revival of spawning after the spawning season had apparently ended is surprising. The quantity of eggs shed is equal to mid-season spawning.

V. 9/2. 4 females spawned in quantity, 2 maximum, 2 one half maximum. 5 males shed sperm, 3 heavy and 2 light. 9 did not spawn.

* September 5, full moon.

VI. 9/5. 6 females shed eggs, 4 of them maximum. 5 males shed sperm, 3 abundantly and 2 light. 4 did not spawn.

VII. 9/6. 6 females spawned, 4 maximum, 2 one half maximum. 5 males shed sperm, none maximum, mostly light. 8 did not spawn.

Note.—This cessation or reduction of spawning in August is typical as shown by five years' experience. Spawning is also regularly revived in September. It is most marked when full moon falls during the first week of the month; and less marked when it is near the middle of the month. This record for 1922 led the writer to study these phenomena in detail in succeeding years. They can scarcely be explained on any other ground than a lunar cycle effect.

SPAWN1NG EXPERIMENTS. 1924.

CUMINGIA TELLINOIDES.

Table No. II.

* July 2, new moon.

I. 7/5. 6 females spawned well (maximum). 5 males shed well (maximum). One did not spawn.

II. 7/8. 4 females spawned well (maximum). 5 males shed well (maximum). 3 did not spawn.

* July 9, first quarter.

III. 7/9. 100 per cent. spawned. 3 females spawned, 2 heavy, one light. 4 males shed, 2 heavy, 2 light.

Remark.—Heavy spawning activity during the past week and nearly 90 per cent. of the individuals spawning approximately maximum quantity.

IV. 7/12. 1 female spawned small amount 1/2 maximum. 4 males spawned, 1/2 maximum. 10 did not spawn.

V. 7/14. 6 females spawned, one heavy, 2 medium and 3 light. 9 males spawned, one heavy, 4 medium, 4 light. 10 did not spawn after hours.

Comment.—Light spawning activity compared with ten days ago which was remarkably heavy. Tendency to spawn reluctantly, some individuals spawning only after hours. One spawned after two hours, others slow to act. Only one good lot of eggs from twenty-five individuals, and none on July 12.

* July 16, full moon.

VI. 7/19. 4 females shed, 2 maximum, 2 light. 5 males shed, 3 maximum, 2 light. 6 did not spawn.

Comment.—Impression of light or medium spawning activity.

* July 23, third quarter.

VII. 7/25. 12 females shed, 5 maximum, 3 medium, 4 light. 7 males shed. Some very heavy, some light. 5 did not spawn.

Comment.—Impression of heavy spawning activity.

VIII. 7/31. 7 females shed, 4 heavy, one medium, 2 very light. 7 males shed, 5 heavy, 2 light. 2 did not spawn.

Comment.—Impression of heavy spawning activity, but not quite as heavy as at last new moon.

* *July 31, new moon.*

IX. 8/2. 5 females shed, 3 heavy, 2 light. 6 males shed, 4 heavy, 2 light. 7 did not spawn.

Comment.—Only one female spawned within an hour. And only two males within the first hour. (Why?) No others spawned for two and one half hours. Impression of poor spawning activity, reluctant and evident retention of eggs from some cause, also low percentage spawned after hours.

* *August 7, first quarter.*

X. 8/12. 11 females shed, 5 near maximum, 2 medium, 4 light. 14 males shed, all heavy or medium. 2 did not spawn within the first hour.

Comment.—This lot began to spawn after fifteen minutes, males more quickly than females. Most of the males began before thirty minutes. Some females began after twenty-five minutes but most of them after 40 minutes. Fourteen males and eight females had shed within one hour. Three females spawned after one and one half hours.

Impression of good heavy spawning activity and the reluctance observed a week ago was gone. The spawning was unusually prompt and vigorous. Cause?

* *August 14, full moon.*

XI. 8/16. 100 per cent. spawned. 6 females shed, 4 heavy, 2 medium. 3 males shed, 2 heavy, one medium. 1 spawned after 1½ hours heavily.

Comment.—Very heavy spawning activity. All except one spawned within an hour. Most of them began in 20 to 30 minutes. The last one spawned heavily after one and one half hours.

Heilbrun collected twelve Cumingia this date. All but one spawned.

XII. 8/20. 6 females spawned within an hour, 4 heavy, 2 medium. 13 males shed, 7 heavy, 3 medium, 3 light. 2 females spawned after 1½ hours. Very few eggs from these two. 1 did not spawn.

* *August 22, third quarter.*

XIII. 8/26. 100 per cent. spawned. 12 females shed during first hour, 5 heavy, 2 medium and 5 a mere trace. 9 males shed, 5 heavy, four light. 2 females spawned after one hour and forty minutes. The last two spawned very few eggs.

Comment.—Impression of heavy spawning activity during the past ten days. Five females gave only a very few eggs which indicates recent spawning on their part at the full moon period.

The inhibition to spawning noticed earlier is not now working. The heaviest spawning of the year has occurred during the past ten days.

XIV. 8/29. None spawned during the first hour. 4 females spawned a few eggs later, 1/10 maximum. 2 males shed a trace of sperm. 14 did not sperm.

XV. 8/30. 2 females spawned. Very few eggs, approximately 1/15 maximum. 3 males shed lightly, 1/10 maximum. 15 did not spawn.

* *September 1, new moon.*

XVI. 9/2. 5 females shed a few eggs, scarcely visible in the dish (a trace). 2 males made water slightly turbid with sperm. 18 did not spawn.

Comment.—Spawning negligible. Experiments for 1924 ended at this time. Experience has shown that spawning no doubt revived at the approach of the September full moon for a few days. This series of experiments shows that spawning was heavy early in July from the time the experiments of the season

began until after the first quarter when they suddenly fell off not to be fully revived until somewhat after full moon. Then followed a week of heavy spawning until new moon.

Shortly after the first quarter in August the heaviest spawning of the year began and continued until the approach of the next new moon or from August 7 to August 29. Then came the usual late season, temporary cessation of spawning which presumably revived somewhat at the September full moon.

It is unfortunate that these experiments could not have begun by the middle of June and continued until the middle of September. The experience of several years leads one to believe that spawning began at full moon in June and reached maximum late in June. The variations in spawning activity under laboratory conditions show that the production of the gametes is greatest around full moon and least around the first quarter. These results are shown regularly except at the height of the breeding season when it may be masked by the rapidity of egg production.

SPAWNING EXPERIMENTS. 1926.

CUMINGIA TELLINOIDES.

Table No. III.

The following table of experiments shows the duration of the spawning season and variations in spawning activity during the lunar cycle. From fifteen to twenty-five individuals were used in each experiment. They were isolated in stender dishes containing enough water to cover them fully. Spawning usually occurs within an hour. Collections were always made at low tide.

- I. 3/1. No eggs and no spermatozoa.
- II. 4/1. No eggs and no sperm.
- III. 6/1. No eggs and no sperm. (Experiment by Hugh Montgomery.)
- IV. 6/11. No eggs and no sperm. (Experiment by Hugh Montgomery.)
- V. 6/15. No eggs, one male shed active spermatozoa, one fifth maximum.
- * June 18, first quarter.
- VI. 6/21. 2 females spawned, approximately 1/5 maximum. 2 males shed sperm, 1/4 maximum. 8 did not spawn.

Comment.—Eggs mature, cleavage normal. Spawning season opened between June 15 and June 20.

- * June 25, full moon.
- VII. 7/1. 6 females spawned, 1/4 to 1/3 maximum. 7 males shed sperm, 1/3 to 1/2 maximum. 2 did not spawn.

* July 2, third quarter.

- VIII. 7/6. 9 females spawned rather abundantly, 1/2 to 3/4 maximum. 12 males shed sperm, 11 heavy, 1 light. 2 did not spawn.

* July 9, new moon.

- IX. 7/15. 2 females spawned, 1/2 to 2/3 maximum. 12 males shed sperm, 10 medium, 2 light. 12 did not spawn.

Comment.—Light spawning, and many failed to spawn.

* July 17, first quarter.

- X. 7/19. 2 females spawned 1/20 maximum (very few eggs). 4 males shed sperm, very light, 1/10 to 1/20 maximum, scarcely detectable in the dish. 13 did not spawn.

Comment.—Spawning all but extinct. Not even one fair lot of eggs obtained. Scarcely visible in the dish. Almost no spawning activity during the past week.

XI. 7/24. 13 females spawned lightly, $1/2$ to $1/3$ maximum. 7 males shed sperm, 6 heavy, 1 light. 2 did not spawn.

Comment.—Great revival of spawning but light in amount.

* *July 25, full moon.*

XII. 7/26. 8 females spawned, 4 heavy (maximum) and 4 medium ($1/2$ to $2/3$ maximum). 12 males shed sperm, 6 heavy, 4 medium, and 2 light. 6 did not spawn.

Comment.—Very heavy spawning. The heaviest this year to date. All spawned promptly after thirty or forty minutes. Lunar periodicity is demonstrated clearly. Almost complete cessation of spawning ten days before full moon but came on again actively at full moon.

* *July 31, third quarter.*

XIII. 8/4. All spawned actively, 100 per cent.

* *August 8, new moon.*

XIV. 8/10. 6 females spawned, 3 heavy, 2 medium, 1 light. 11 males shed sperm, 10 heavy or medium, 1 light. 6 did not spawn.

XV. 8/13. 7 females spawned, 6 medium or heavy, 1 light. 7 males shed sperm, mostly heavy. 1 did not spawn.

XVI. 8/16. 100 per cent. spawned. 8 females spawned in quantity. 7 males shed sperm in quantity.

* *August 16, first quarter.*

XVII. 8/20. 5 females spawned ($1/2$ maximum or less). 7 males shed sperm, $1/2$ maximum. 2 did not spawn.

XVIII. 8/21. 6 females spawned heavily, $1/2$ to $3/4$ maximum. 9 males shed sperm heavily. 5 did not spawn.

* *August 23, full moon.*

Comment.—There is no evidence of reduced spawning at the first quarter in August, whereas in July there was a clear-cut case.

XIX. 8/28. 7 females spawned, approximately $1/2$ maximum. 12 males shed sperm, medium to light. 5 did not spawn.

Comment.—Spawning at this time by about 80 per cent. of the individuals but for the most part below the spawning of last week in quantity. Good lots of eggs still obtained, $1/2$ to $1/3$ maximum the rule. The quantity of eggs spawned by each individual is usually reduced toward the end of the season.

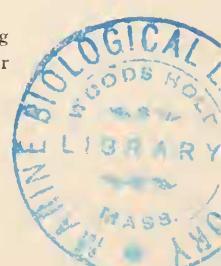
* *August 29, third quarter.*

XX. 8/31. 3 females spawned, $2 1/10$ maximum, $1 1/20$ maximum. 4 males shed sperm ($1/4$ maximum). 25 did not spawn.

XXI. 9/1. 6 females spawned $1/20$ maximum (scarcely visible in the dish). 4 males shed sperm, very light, $1/10$ maximum, water only slightly turbid. 14 did not spawn.

XXII. 9/3. No eggs and no sperm. Some extended the siphons and lashed them in characteristic manner but there were no eggs in the ducts.

Comment.—Very light spawning. Almost none since August 28. Spawning suddenly fell off, one week after full moon. Vigorous spawning continued for five days after full moon.



* September 7, first quarter.

XXIII. 9/9. 6 females spawned, 1 2/3 maximum, 4 1/5 maximum, 1 1/10 maximum. 5 males shed sperm, 3 1/2 maximum, 2 light. 19 did not spawn.

Comment.—Considerable revival of spawning noted. Much more active than last week, but still relatively insignificant. Good for so late in the season.

* September 21, full moon.

XXIV. 9/21. 3 females spawned, 1 maximum, 1 1/5 maximum, 1 1/10 maximum. 2 males shed sperm, 1 3/4 maximum, 1 1/2 maximum. 15 did not spawn.

Comment.—Revival of spawning evident, though not extensive. Quantities surprisingly large. Temperature 18° C., or 17½° C.

XXV. 9/10. Temperature 17° C. No eggs and no sperm.

Comment.—The spawning season of *Cumingia* ends between September 15 and October 1. This year spawning continued until the end of September. The foregoing data give no very definite information about the time of spawning in nature. It is evident that eggs are produced almost continuously although probably not uniformly. There is a lunar periodicity either in the production of the gametes or in the spawning or in both. Spawning by each individual occurs more than once during the season.

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CYTOTOLOGY OF *SACCHAROMYCES CERVICIAE* WITH ESPECIAL REFERENCE TO NUCLEAR DIVISION.

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For some years following the discovery of cell division it was thought that direct division was responsible for the duplication of the nucleus, and even after the discovery of indirect division the former was still considered to be the usual method and mitosis was held to be a peculiar and infrequent phenomenon. Some held that the two processes were related and that mitosis was derived from the simpler and more primitive one, amitosis. However, as the investigation of this problem was continued evidence was gradually accumulated to show that mitosis is the usual if not universal method of cell division in higher animals and plants, and that amitosis is not a reproductive phenomenon at all, but has for its function the increase of nuclear surface in relation to volume and is rarely, if ever, followed by cytoplasmic division. This conception was well expressed by Conklin (1917) in the following passage, "Mitosis and amitosis are fundamentally unlike. Mitosis is the one and only method of bringing about equal division and distribution of the chromatic material of the nucleus. Amitosis is not a genuine divisional phenomenon at all, but merely a means of increasing the nuclear surface and of distributing nuclear material throughout the cell, comparable to nuclear lobulation, fragmentation or distribution. These two processes are not equivalent or even comparable, nor may one of them be converted into the other." Although this view is not universally held it is very generally accepted by cytologists at the present time.

The study of cell reproduction is much more difficult in Protozoa than in higher forms, as many of the types of intra-nuclear division have a superficial resemblance to amitosis. Minchin (1912) actually accredits the description of direct division in a number of protozoa, although he admits that some

of the formerly supposed cases of amitosis have been shown to be merely simulations of it. Some of the authorities in this field, however, are willing to elevate the Protozoa to essentially the same position as Metozoa in this respect. Kofoid (1923) says: "In the first place amitosis as described in the Protozoa is either a pathological or degenerative process, as it is in the Metozoa, or it is based on a partial account of the normal process of mitosis in which the nuclear membrane remains intact throughout the whole process, as it does in the flagellates and rhizopods, and in its anaphases presents a superficial resemblance to pathological amitosis. The persistence of the nuclear membrane in no way interferes with the occurrence of chromosomes constant in number and kind. In other words the doctrine of chromosome continuity, in so far as amitosis is concerned, is no more affected in the Protozoa than in the Metazoa." The nuclear division (promitosis) of many of the lower protozoa is unquestionably very different from the mitotic division exhibited by higher plants and animals, but it seems likely that in all cases it is a simplified form of mitosis and is entirely unrelated to amitosis, even though it does superficially resemble it.

It seems as though the difficulty of studying nuclear division in any form is responsible for the persistence of investigators in describing it as amitotic. The tapeworm *Monezia* offers an example of this (Child, 1911). Yeast belongs in the same category, and we should realize the extreme difficulty of investigating this problem in yeasts by considering the minute size of the cells and the fact that for some time there was a heated controversy as to whether or not they even possess a nucleus. Some of those who took the affirmative, as has since been proved, were describing structures which belong to the cytoplasm. Wager (1898) and Wager and Peniston (1910) described as the nucleus, the actual nucleus, the vacuole, and a part of the metachromatic material surrounding the nucleus and vacuole. The division of this compound structure was described as amitotic in the case of budding and by an "intermediate step in karyokinesis" in the case of spore formation. This account of the indirect division of a cytoplasmic vacuole is comparable to the early figures of mitosis of the parabasal body (kineto-

nucleus) in trypanosomes and shows that without a favorable modification of technique the problem is almost invincible.

Some of the early contributors to our knowledge of the cytology of yeasts unquestionably saw and illustrated, with a fair degree of accuracy, not only the resting nucleus but also stages in its division. Janssens (1902) and Janssens and Leblanc (1898) considered the division of the nucleus to be an intermediate form of mitosis. Swellengrebel (1905) and Fuhrmann (1906) as a true mitotic process. Although these last two articles are in the main correct they have not been generally approved, and the ideas of Guilliermond (1904, '12, '17 and '19), which gain weight by the mere bulk of his work on yeast, seem to meet with more favor. This author, who is responsible for a large part of our knowledge of the well developed sexuality of yeasts and for an excellent account of the typical metazoan mitosis found in spore formation in *Schizosaccharomyces octosporus*, maintains that in bud formation the nucleus divides by a process identical with amitosis in the tissue cells of higher organisms, where, as Conklin asserts, it is not a reproductive phenomenon at all. To accept this would be to admit that mitosis and amitosis are fundamentally alike and interchangeable. This would undermine a large part of our knowledge of cytology and genetics. The problem most assuredly warrants critical study.

METHODS.

Pure cultures of *Saccharomyces cerviciae* were used for this work. They were cultivated on both liquid and solid media. French proof broth was used for the liquid medium, French proof agar for the solid.

The organisms were transferred to slides which had been previously smeared with albumen fixative and the moist films were fixed either in corrosive-acetic-alcohol (95 per cent. alcohol saturated with mercuric chloride 95 parts, glacial acetic acid 5 parts) or Bouin's solution (saturated picric acid solution 75 parts, formalin 20 parts, glacial acetic acid 5 parts). Iron-alum-hæmatoxylin counterstained with light green or not counterstained at all was found to be the best means of staining. Delafield's hæmatoxylin and carbol fuchsin were tried without

success, and eosin and orange G were found to be equally useless.

Fixation in corrosive-acetic-alcohol slightly shrinks the cells and gives the chromatic material in the cytoplasm such great affinity for basic stains that in most cases the nucleus is obscured. After fixation in Bouin's it does not stain so heavily and the nucleus can be seen in all cases, providing it is not too heavily stained. The successful technique finally developed was fixation in Bouin's and staining before the picric acid was completely washed out. This decreased the affinity of both nuclear and cytoplasmic chromatic material for stain, especially the latter. In this way the nucleus could be stained without showing the chromatic bodies in the cytoplasm at all. This procedure made possible a careful study of the nuclear chromatin both at rest and during division. Light green was found very valuable for the study of the metachromatic granules. Even when they are very abundant this stain will demonstrate their granular nature. In preparations in which no counterstain or the other acid stains are used they frequently appear as a single, large mass.

It was found that fixation and staining in carbol fuchsin followed by light green furnished an excellent method for demonstrating the alveolar nature of the cytoplasm. Although the nucleus could be seen in these cells its minute structure was not visible and the method is of no value for the present work. Light green is taken up by the gelatinous secretion sometimes given off by the yeast cells and consequently furnished a good means of demonstrating the presence of this substance.

MORPHOLOGY.

Saccharomyces cervicæ is a round to slightly oval yeast whose size ordinarily ranges from 5 to 10 micra. In old cultures the cells are occasionally elongated, sometimes crescentic (Fig. 7). The cell is enclosed in a thin though distinctly evident wall. This wall occasionally becomes greatly thickened and the cells pass into a very resistant stage (durable cells, chlamydospore) such cells can be kept dry for a year without destroying their viability (Figs. 10 to 13).

One of the most prominent structures inside the cell is the

vacuole, small or entirely absent in very young cultures, but attaining a relatively great size after several days. In some cases it almost entirely fills the cell (Fig. 2). This vacuole is generally round, with a very regular outline. The cytoplasm is distinctly alveolar and contains numerous metachromatic granules. Occasionally cells are found with none of these granules, especially in very young cultures (Fig. 5) and sometimes there are only a few present, in which case they are located near the nucleus (Fig. 4). In old cells with a large vacuole there is frequently such a great mass of metachromatic material surrounding the nucleus that the latter body is obscured (Figs. 2 and 3), and the granular nature of the metachromatin is not discernible. Lines of fine granules can be seen leading from this mass to large granules in other parts of the cell, thus converting the metachromatic material into a connected unified system. These granular strands are especially evident where they go around the vacuole, and constitute the nuclear reticulum of Wager. Guilliermond figures basophilic granules within the vacuole. It is these granules above and below the vacuole which he sees and there are really no granules within the large vacuole. When material is fixed in Bouin's solution and stained in iron-alum-haematoxylin without washing out the picric acid the metachromatic granules do not stain. When this procedure is properly carried out, the well stained nucleus embedded in the alveolar cytoplasm is quite evident and easily studied. (Figs. 6 to 8.)

The nucleus of *Saccharomyces* is quite similar to that of higher plants, particularly *Phaseolus* (Kater, 1926). The nuclear membrane is equally as evident as in higher plants. Centrally located is a large basophilic nucleolus or karyosome from which radiate slender slightly basophilic linin strands. These strands run from the nucleolus to the nuclear membrane in identically the same manner as in *Phaseolus*. There are generally about six such strands visible. Just inside the nuclear membrane are located a number of chromatin granules. The larger ones are found at the points where the linin strands come in contact with the nuclear membrane. Except for the minute size of the nucleus (1 to 3 micra in diameter) it is hardly distinguishable

from that of *Phaseolus*. A fruitful study of the finer points of the structure of the resting nucleus as well as following it through division would have been impossible without some means of staining it without affecting the metachromatin. It is this feature of our technique which makes the observations convincing. A centrosome could not be identified in the interkinetic cell nor could any variation in the cytoplasm surrounding the nucleus, comparable to sphere substance, be seen.

The yeast cell contains both fat and glycogen, the former usually in small globules in the vicinity of the nucleus.

It has been previously observed that yeast cells will give off a gelatinous secretion when permitted to dry gradually in a closed vessel. This is thought to play a part in the agglutination of yeast and the consequent clearing of the medium. In the present work it was found that dilution of the medium of an old culture with distilled water will produce this secretion (Fig. 9). The secretion has great affinity for light green.

BUDDING.

In young cultures the usual method of reproduction is by budding. The superficial features of this process are matters of common knowledge, and consequently, this description will be limited to the internal phenomena. The bulge in the cell wall and the entrance of cytoplasmic elements into the bud occurs some time before any change in the resting nucleus is observable (Fig. 14). The finely vacuolated cytoplasm is the first material to enter the bud. This is followed by the metachromatic granules. In those cells that contain a large vacuole the bud is generally formed near the nucleus and consequently near the greater part of the metachromatic material. The mass of this material near the bud separates into individually visible granules, part of which migrate through the isthmus into the bud, the rest moving to the opposite side of the parent cell (Figs. 15 and 21). Whether or not they divide at this time cannot be stated. Even after these granules are distributed to the two cells the nucleus is still in the resting condition. The large clear vacuole does not divide, but after the bud has attained almost the size of the parent cell a small vacuole appears within it and gradually enlarges.

After the bud has attained almost normal size and the distribution of metachromatic granules is completed the nucleus gives evidence of impending division. The chromatin accumulates on the linin strands, making them very evident and extremely basophilic. At the same time the disperse chromatin granules enlarge and the whole nucleus becomes a perfect miniature of an early prophase in *Phaseolus vulgaris* (Kater, 1926, Figs. 15 and 16). Meanwhile the nucleus remains stationary (Fig. 16). The steps in the transformation of such a nucleus into chromosomes cannot be followed with any degree of certainty, but many stages such as the one represented in Fig. 17 were seen. A knot of chromosomes probably occupying the old location of the nucleolus is here observed at the periphery of which individual chromosomes are becoming disentangled from the rest.

So far neither a dividing centrosome or spindle can be identified. However, as the chromosomes become arranged on the metaphase plate a spindle becomes visible (Fig. 18) and in some cases the ends are lodged in a granule perfectly comparable to the centriole in metazoa. No astral radiations are visible (Fig. 19). Such a centrosome has previously been reported in yeast (Swellengrebel, 1905; Guilliermond, 1917). In Fig. 18, the chromosomes are quite clearly separating in the same way as they do in higher plants and animals. Except for the difference in size the early anaphase represented in Fig. 19 could easily be mistaken for a metazoan mitotic figure. No careful attempt was made to count the chromosomes of *Saccharomyces* as their size makes it impractical. However, it seems certain that there are more than the four reported by Swellengrebel (1905) and Fuhrmann (1906). Probably at least twice that number.

The migration of the chromosomes through the isthmus into the bud is not at all clear. In some figures the orderly arrangement of the anaphase plates seems to be maintained (Fig. 22) while in others the chromosomes appear to enter the bud one at a time. In such cells the spindle disappears and only the irregularly arranged chromosomes can be seen (Fig. 21). In overstained slides specimens similar to the one illustrated in Fig. 22 very greatly resemble amitosis, and are probably responsible for the confusion on this point.

After being distributed to the two daughter cells the chromosomes become collected at one point and apparently fuse forming a uniformly basophilic mass in which the individual chromosomes cannot always be identified. The imbibition of achromatic material results in the alveolization of the outer part of this mass. The limiting membrane (nuclear membrane) is frequently bulged between the linin strands (Fig. 25). A continuation of alveolization results in a spherical nucleus with a nucleolus of moderate size, the resting condition.

The division of the cytoplasm in the isthmus occurs shortly after the separation of the chromosomes and the disappearance of the spindle (Fig. 24). Although the cytoplasm has separated, the cell wall frequently does not divide for a considerable length of time and holds the two cells together. In this way a number of cells are sometimes connected, somewhat resembling a mycelium (Fig. 8).

No attempt has been made to study the formation of ascospores, but according to previous accounts the nucleus divides by mitosis.

DISCUSSION.

The above account leaves two points without adequate solution, namely the transformation of the nucleus into chromosomes and the migration of chromosomes through the isthmus. Fortunately these are matters of detail and the remainder of the account shows conclusively that the nucleus of yeast, in the formation of buds, does not divide by constriction, but that chromosomes are formed, divide (Fig. 18), separate, and give rise to daughter nuclei in the same way as in higher organisms, and yeast can be placed in the same category with higher animals and plants, and protozoa as enunciated by Conklin and Kofoid respectively.

To accept Guilliermond's descriptions of the indirect division of the nucleus in the formation of ascospores and direct division in budding would be to admit the reproductive nature of amitosis and, secondly, that a nucleus produced by this method may later divide, in the ascus, by perfectly normal mitosis. This would make untenable many of the generalized conceptions of

the cytologist and the geneticist. It seems remarkable that the investigation of nuclear division in yeast has not previously attracted the attention of cytologists, as well as mycologists.

In the earlier work on *Phaseolus* it was found that the linin strands radiating from the nucleolus are actually the linin sheaths of chromosomes which persist through the resting condition and give the chromosomal vesicles morphological individuality during interkinesis. It seems probable that the linin strands of *Saccharomyces* are of the same nature. The collection of chromatin along them in the early prophase (Fig. 16) and the bulge between them in the telophase (Fig. 25) would indicate this. However, since the actual transformation of the prophase nucleus into chromosomes and the alveolization of early telophase chromosomes could not be followed with any degree of certainty a definite statement of chromosomal continuity in yeast cannot be made on a morphological basis as in *Phaseolus*, but the probable homology is certainly worthy of mention.

The existence of radiating linin strands alone would not justify this interpretation, since they are present in many protozoan nuclei where the nuclear membrane remains intact throughout mitosis. In such organisms the membrane is not a product of the linin sheaths of chromosomes, as in higher animals and plants, and the linin strands could not easily be interpreted in the same manner. Such a nucleus is found in *Polytomella citri* (Kater, 1925). In *Saccharomyces* the nuclear membrane disappears and consequently it is quite possible that the linin strands are homologous with those of *Phaseolus*.

SUMMARY.

The nucleus of *Saccharomyces cerviciae* divides by mitosis in the process of budding.

The chromosomes apparently form, divide, separate, and give rise to daughter nuclei in much the same way as in *Phaseolus*. The linin strands connecting the nucleolus and nuclear membrane probably represent sheaths of chromosomal vesicles.

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EXPLANATION OF PLATES.

Figures 1 to 5 and 15, 20, and 21 made from material fixed in corrosive-acetic-alcohol and stained with iron-alum-hæmatoxylin, Figs. 4, 5, and 15 counterstained with light green. The remaining figures from material fixed in Bouin's solution and stained as above. All drawings made with Abbe model camera lucida. Magnification 3,200 \times .

PLATE I.

FIG. 1. Small yeast cell showing fine granular lines of chromatic material in the cytoplasm, nucleus partly obscured.

FIG. 2. Nucleus entirely obscured. Granular lines above vacuole clear.

FIG. 3. End view of a cell similar to Fig. 2.

FIG. 4. Cell containing only a few metachromatic granules. Nucleus visible. Medium-sized vacuole.

FIG. 5. No metachromatic granules. Nucleus very large and structure clear.

FIG. 6. Nucleus above vacuole which is not so clear as in side view.

FIG. 7. Cell exhibiting crescentic form.

FIG. 8. Four connected cells showing manner of connection by unbroken wall.

FIG. 9. Cross hatching indicates gelatinous secretion which holds the cells together.

FIG. 10. Resistant cell. Note heavy wall and abundance of metachromatic granules.

FIG. 11. The same. Metachromatic granules disappeared.

FIG. 12. Resistant cell showing departure from spherical form and shrinking of protoplast.

FIG. 13. Resistant cell that has been kept dry for one year.

Drawings by G. T. Kline.

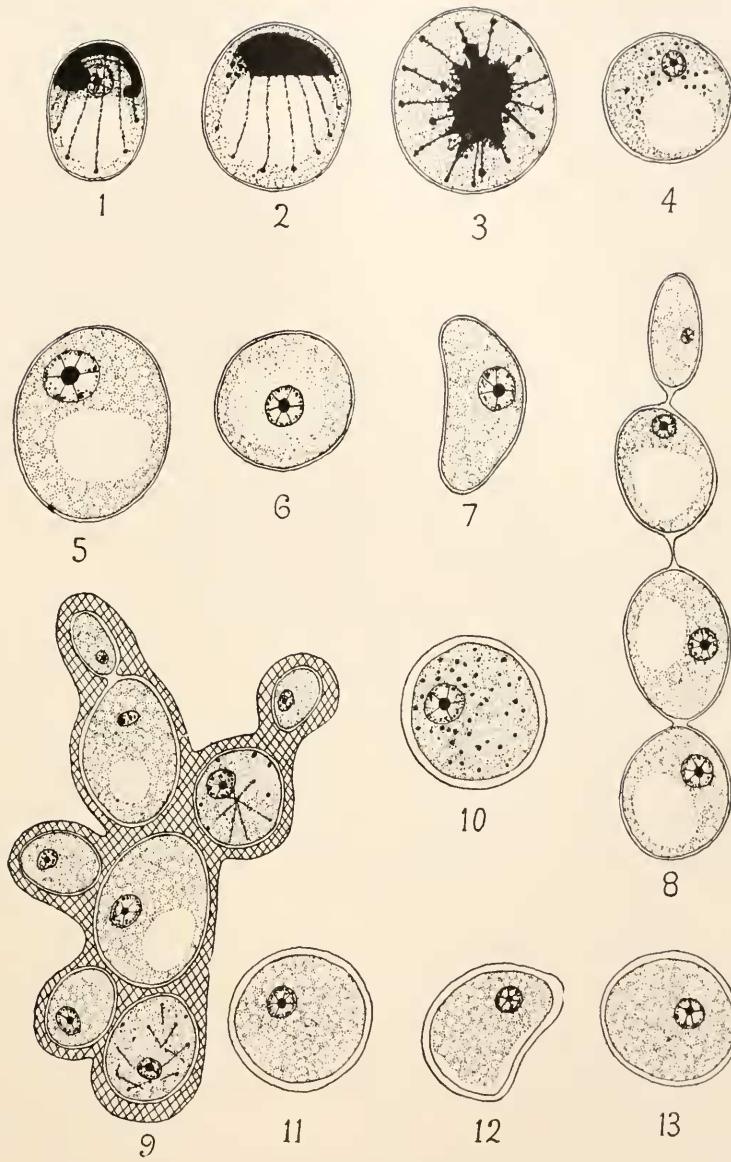


PLATE II.

FIG. 14. Early bud. Extremely large vacuole.

FIG. 15. Metachromatic granules entering the bud. Nucleus not yet beginning division.

FIG. 16. Early prophase. The chromatin is collecting on the linin strands. A few metachromatic granules visible in both cells.

FIG. 17. The nucleus is breaking up into chromosomes. Slightly oblique view making bud appear abnormally small.

FIG. 18. Metaphase. The chromosomes are separating on the equator of the spindle.

FIG. 19. Anaphase. Centrosome and spindle very evident. Metachromatic granules in both cells.

FIG. 20. The same. Slightly later.

FIG. 21. The chromosomes are passing through the isthmus. A few metachromatic granules in opposite ends of both bud and parent cell. These are much smaller than the chromosomes.

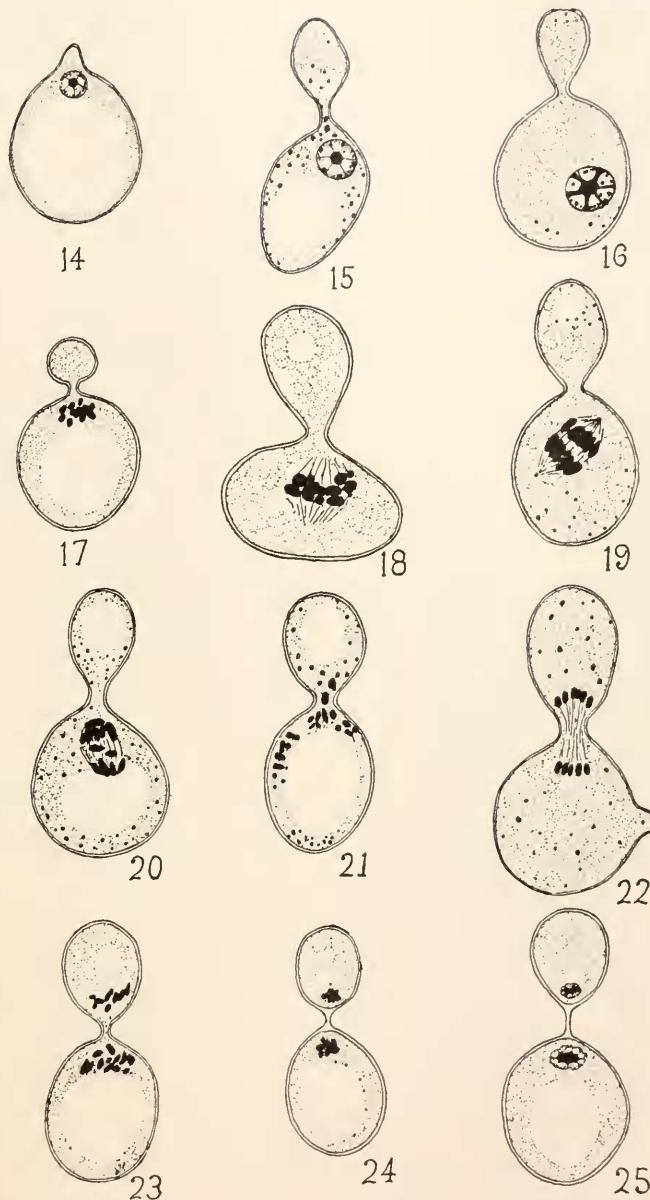
FIG. 22. Late anaphase. Spindle very evident. A second bud on the parent cell.

FIG. 23. The spindle not visible. The isthmus is closing. Chromosomes collected near one point.

FIG. 24. The cytoplasm has divided, the cells being held together by their walls. Chromosomes beginning the apparent fusion.

FIG. 25. Late telophase. Large nucleolus. Note bulges in nuclear membrane between linin strands.

Drawings by G. T. Kline.



MEASURES OF INSECT COLD HARDINESS.

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Different measures of insect cold hardness used by different workers may usually be reduced to the empirical survival test. Bachmetjew (1901) used the "vital temperature maximum" or the second time an insect reached the undercooling point. Duval and Portier (1922) considered that there was a freezing point below that ordinarily determined, the higher freezing point being that of the body fluids, the lower that of the body cells.

In strong contrast to the scarcity of measurements of insect cold hardness, stand the many determinations by plant physiologists. Osmotic pressure as determined by freezing point lowering has been widely used from the time of Sachs and Pfeffer. Water content has been of value as a criterion of cold hardness in plant groups far separated taxonomically. For example, Johnson (1923) used water content of peach buds as a measure of cold hardness, and Steinbauer (1926) employed it for clover seeds. Newton and Gortner (1922) and Newton (1924) emphasize the importance of bound water to cold hardness. Müller-Thurgau (1886) proved conclusively that some plants could survive freezing. The ability of a plant to survive freezing was defined by Harvey (1918) as cold hardness.

The two kinds of insect cold hardness (1) hardness to the quantity factor of low temperature or ability to withstand long periods of relatively mild low temperature and (2) hardness to the intensity factor of low temperature, or ability to withstand extremes of low temperature have been discussed in a previous paper. In the present paper cold hardness to the intensity factor alone will be considered.

Closely associated with changes in cold hardness are changes in moisture content. Insects dehydrated but not to the period of injury, can withstand temperatures far lower than undehydrated individuals. This is strikingly true for insects that are

not self dehydrating to any large extent. Thus the Japanese beetle, *Popillia japonica* Newm., does not exhibit any marked body weight changes over winter when kept in moist surroundings, but can be experimentally dehydrated to half its body weight. When thus treated they are very cold resistant, having a survival temperature of as low as -28°C . In contrast

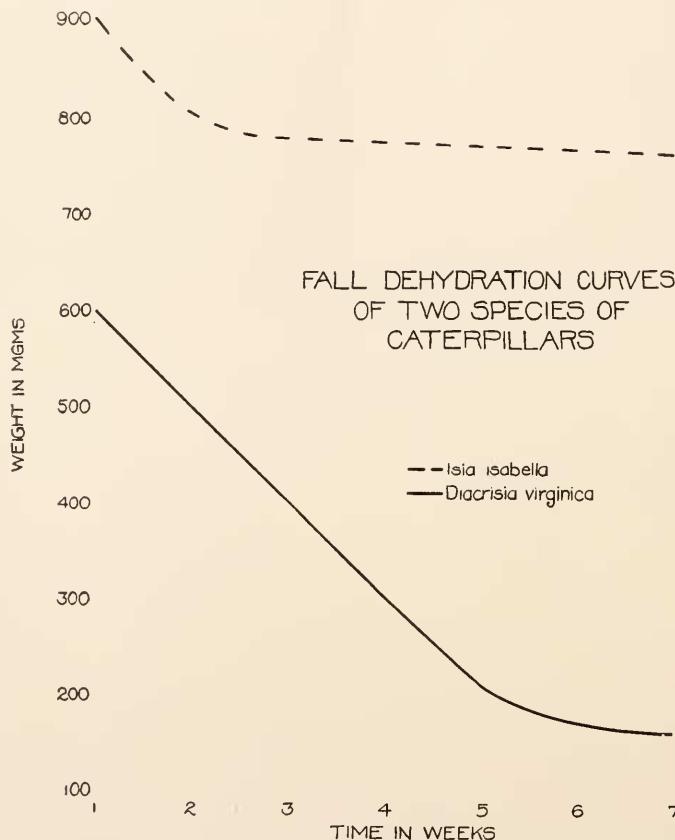


FIG. 1. Fall dehydration curves of two species of caterpillars, *Isia isabella* and *Diacrisia virginica*.

to the Japanese beetle larvæ there are some species of oak borers and caterpillars which are normally self dehydrating during the winter. The dehydration curves (Fig. 1) of *Isia isabella* Hy. Edw. and *Diacrisia virginica* Fabr. show a marked water loss as these caterpillars go into hibernation. At the period of inflection

of the weight loss curve (Fig. 1) these insects can survive freezing. When the curve is plotted with rate against weight loss the point of inflection is brought out more clearly (Fig. 2). Up to

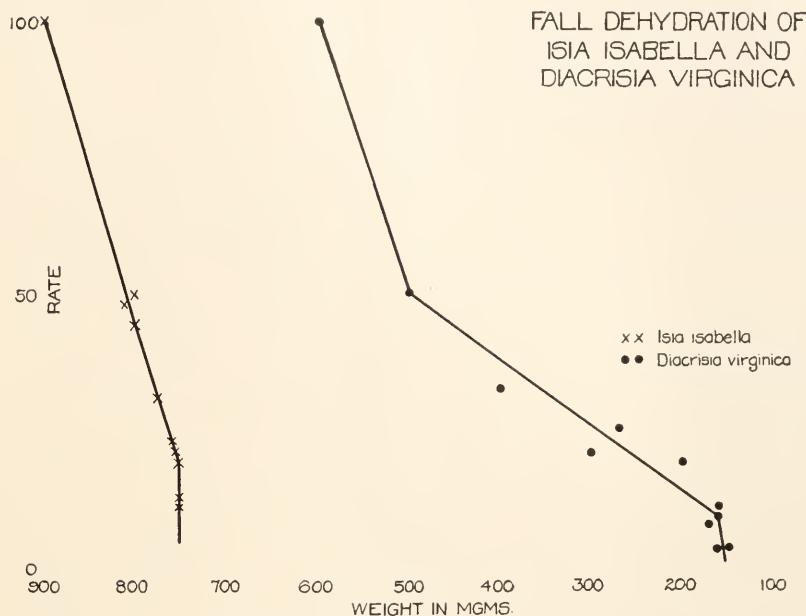


FIG. 2. Fall dehydration of *Isia isabella* and *Diacrisia virginica*. $\frac{1}{\text{time}}$ or rate plotted against weight.

the point of inflection of the weight loss curve the undercooling point of the blood is the minimum survival temperature. Beyond that point the undercooling point no longer measures the total cold hardiness which reaches to below -40° C. There is no free body fluid on which a conductivity reading can be made.

The oak-borers, *Synchroa punctata* Neum., *Dendroides canadensis* Lec., *Romaleum rufulum* Hald. also are normally self dehydrating but never to the extent of losing all their free water. Although very cold resistant, having survival temperatures of below -40° C., at no time even in the deepest winter, is it impossible to obtain blood samples. But conductivity is found to be proportional to the survival temperature (Fig. 3). The water content of these insects, obtained by heating them in an oven for four hours at $+50^{\circ}$ C., is only relative but does appear

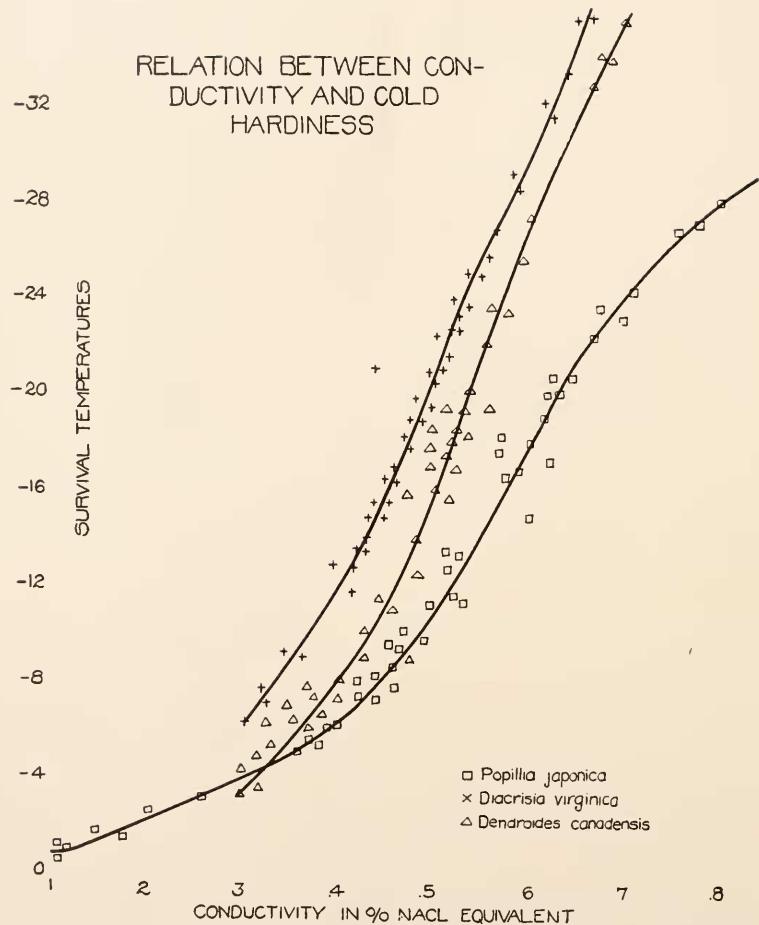


FIG. 3. Relation between conductivity and cold hardiness. *Popillia japonica* □, *Diacrisia virginica* ×, *Dendroides canadensis* △.

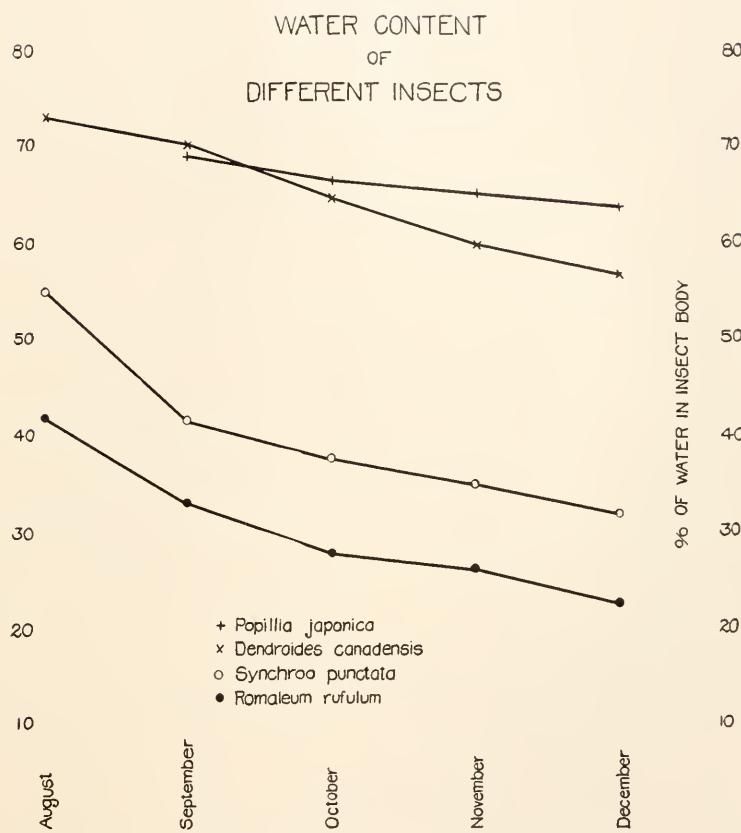


FIG. 4. Water content of different insects during the season at which they develop cold hardiness. *Popillia japonica* +, *Dendrodoea canadensis* X, *Synchroa punctata* o, *Romaleum rufulum* ●.

to give comparable results with different species. The per cent. of water before and during hibernation of three species of oak-borers and of the Japanese beetle are shown in Fig. 4.

The Japanese beetle larvæ, *Popillia japonica* Neum. represent an ecological group far more protected than either the oak-borers or the woolly bear caterpillars. This species hibernates in the ground below the frost line. About 97 per cent. are third instar larvæ and about 3 per cent. second instar. There is a cyclic change in the cold hardiness of these larvæ, not as marked, however, as in the oak-borers but more apparent than in the

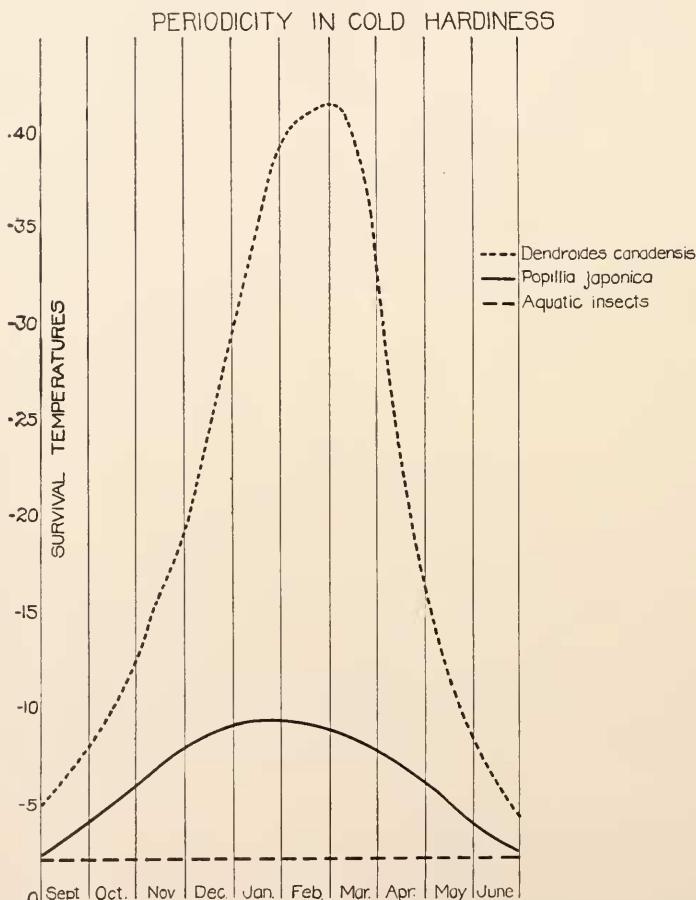


FIG. 5. Periodicity in cold hardiness. *Dendrodoea canadensis* — —, *Popillia japonica* — —, aquatic insects - - -.

aquatic insects where there is practically none, Payne (1926). This periodicity in cold hardiness is shown in Fig. 5. The relation between undercooling and survival temperatures is shown in Fig. 6. Cold hardiness greater than is usually found in their

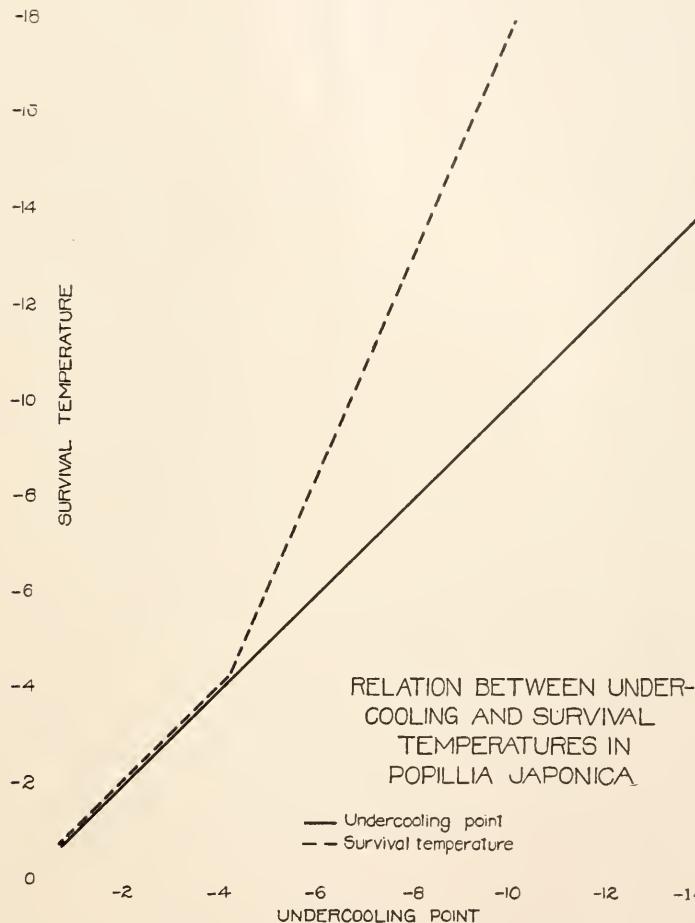


FIG. 6. Relation between undercooling and survival temperatures in *Popillia japonica*. Undercooling point ——; survival temperature ——.

soil habitat can be induced in this insect by dehydration. Conductivity measurements of the blood of dehydrated insects were made. The greatest cold hardiness was found in the dehydrated insects and the least in those infected with wilt disease or

polyhedralskrankheit. In this disease both the freezing point and the conductivity of the blood approach that of water. In Fig. 3 the conductivities of the Japanese beetle larval blood are plotted against survival temperature. Cold hardiness in this species is more fully measured by conductivity than by either moisture content or undercooling point.

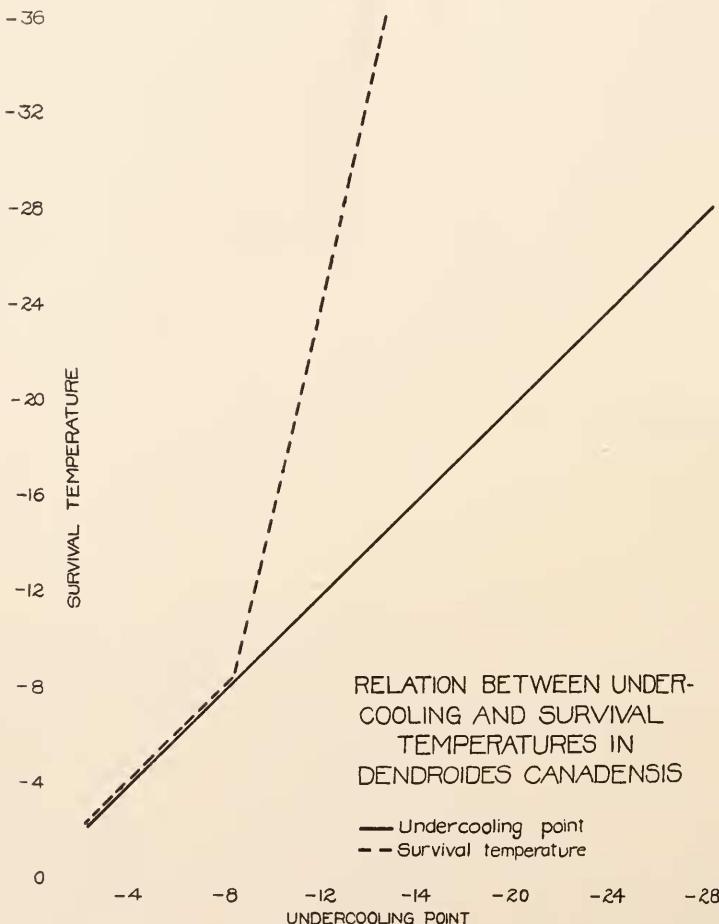


FIG. 7. Relation between undercooling and survival temperatures in *Dendroides canadensis*. Undercooling point ——; survival temperature - - -.

SUMMARY.

1. Cold hardness to the intensity factor of low temperature can be measured by moisture content, undercooling point, and blood conductivity.
2. Up to the time when a given insect can survive freezing, undercooling is a reliable measure of cold hardness. Beyond the point when an insect can survive freezing, undercooling measures but a part of the total cold resistance of a given insect.
3. Conductivity measurements are found proportional to cold hardness throughout the whole year. In some insects there is insufficient free body fluid in winter on which to determine blood conductivity.
4. For each species there is a different set of physical constants which measure the cold hardness of that species.

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COMPENSATORY HYPERSTROPHY OF THE TESTES IN BROWN LEGHORNS.¹

L. V. DOMM AND MARY JUHN.

I. INTRODUCTION.

Compensatory hypertrophy of the surviving testis after unilateral castration was observed as early as 1890 by Ribbert (1). Ribbert worked on mammalian material, using young but almost mature rabbits. Ribbert removed the right or the left testis and then compared the surviving gland with controls of the same age after three months; considerable hypertrophy was almost always found. The hypertrophied testis weighed six times as much as one control gland in three cases. There was no difference in the degree of hypertrophy between the right or the left testis. If the operated animal did not increase in weight as much as did the normal controls, then the retained gland also showed a corresponding lack of development.

Lipschütz '22 (2) repeated these experiments of Ribbert, using also rabbit material for his experiments. It appears to follow from Lipschütz's paper that there is a compensatory increase in weight of the surviving testis after unilateral castration when the operation is performed on young rabbits. This increase in size and weight becomes progressively less the longer the operated animals are kept; about one year after the operation there is not much difference in the weight of the surviving testis and in that of one of the control pair of glands. Unilateral castration is not followed by a significant increase in the size of the remaining testis when the gonad is removed in adult rabbits. Lipschütz believes that his results indicate that the testis is incapable of true compensatory hypertrophy; the actual increase in weight observed being due only to a more rapid rate of growth of the isolated gonad.

¹ From the Whitman Laboratory of Experimental Zoölogy of The University of Chicago. The expenses of this investigation were supported in part by the Committee for Research in Problems of Sex of the National Research Council; grant administered by F. R. Lillie.

The two papers cited above report a certain difference in the results obtained. The discrepancy may be due to the time the surviving gonad was retained and also to the age of the animal at the time of operation.

We became interested in the problem of compensatory hypertrophy of the testis as applied to the material used in the laboratory for a variety of experiments, pure bred Brown Leghorn cocks. The experiments were begun early in July 1924 and terminated at the end of April 1925.

We were guided in outlining the course of the experiments by the following points of view: (a) the appearance of compensatory hypertrophy as such after the removal of one of the pair of gonads; (b) the influence of the age of the birds at the time of the operation on the possible increase in size of the surviving gonad; (c) whether the time that the surviving gonad is retained is of effect on the degree of possible hypertrophy taking place. (d) Finally we wished to determine if there was a significant difference in the amount of the hypertrophy taking place in the right or the left gonad after unilateral castration.

After the experiments were terminated and the data completed we became acquainted with a paper by Benoît '25 (3). This author carried out a series of unilateral castrations on White Leghorns; the operations were performed on three young birds of 18-20 days of age, on one young bird aged two months and on two birds aged seven months each. Control gonad weights were stated for the groups of different ages. According to the results obtained by Benoît, there is a very real increase in the weight of the surviving gonad when castration is carried out at an early date. In the three cases where unilateral castration was performed in baby chicks the surviving testes were retained for about twelve months. At this date each one of the hypertrophied testes weighed approximately as much as, or slightly more than, both testes of the control. The surviving gonad of the cockerel which was operated upon at two months was retained for seven months, at the end of this time it weighed almost 50 per cent. more than the control pair of testes, but it is important to observe that "controls" of this age vary greatly among themselves. Benoît observed no significant hypertrophy

when unilateral castration was performed on birds aged seven months. The surviving testis was retained about a year. Benoît concludes that there is a hypertrophy of the surviving gonad when the one member of the pair is removed in very young birds; unilateral castration of older birds, after the testes have achieved approximately their normal size is not followed by a compensatory increase in the weight of the surviving gonad.

The results published by Benoît agree on the whole with those obtained in this laboratory, but we differ slightly from him in the observations on older birds as will appear in the discussion.

The phrase "compensatory hypertrophy" defines the conception, viz: that loss results in stimulating the growth of the surviving member to an extent that tends to restore a normal quantitative balance between the total gonad tissue and the bird. It involves the corollary that there is a normal quotient for weight of bird divided by weight of gonad tissue. The present study aims merely at testing this assumption. The difficulties arise from the fact that the assumed normal quotient of weight of bird divided by weight of gonad varies (1) with age very markedly; (2) with the time of year, age being the same; and that (3) no organ of the body probably is so susceptible to general conditions of health as the testis. These difficulties create numerous sources of error for any very exact formulation, so that we felt that it was not desirable in the present status of this subject with reference to our main problems to use a sufficient amount of material and time to reach quantitative results. The present study, although it gives positive results, is therefore merely suggestive.

It is a pleasure to express our thanks to Professor F. R. Lillie for his continued interest in the work and for his helpful suggestions during its course.

II. DATA ON UNILATERAL CASTRATION.

All the unilateral castrations were carried out on pure bred Brown Leghorn cockerels that were obtained from one well-known source. The birds were divided into four groups, the first being about one week of age, the second sixteen weeks, the third

twenty-four weeks and the fourth between thirty-two and forty weeks of age at the time of operation.

The operated birds and their controls in each group were hatched at the same time and kept in the laboratory under identical conditions. Comparisons were made only within the groups and in no case between birds of the same age but hatched at different periods.

The cockerels and their controls were weighed at the time of operation and the measurements of the head furnishings and spurs taken. These observations were repeated every eight weeks and a record kept of the condition of the experimental and control birds during the entire experimental period. The testes were removed through an incision between the last two ribs, the gland rapidly weighed and the volume obtained through displacement of normal saline. In the group of baby chicks where the testes were removed at about one week of age, the gonads were not weighed, but the length and width of the gland taken with a pair of fine callipers. The removed testes were fixed in Bouin's fluid at 37° C. and kept in the incubator at that temperature for several hours, varying with the size of the gonad. The usual procedure was followed in washing, etc., and the testes preserved in oil of wintergreen for future histological work.

The greater number of the chicks operated upon at one week of age was lost together with their controls owing to unfavourable weather conditions. The survivors were kept until they were thirty-two weeks of age and then completely caponized or killed.

In the three other groups the right gonad was removed from a certain number of cockerels and the left from a similar number. The surviving right or left testis was then retained for eight, sixteen and twenty-four weeks respectively, at the end of each of these periods one cockerel having a right testis, one cockerel having a left testis and two control birds were completely caponized or killed.

We found in the course of our observations that loss of weight on the part of the cock is reflected in a corresponding diminution of the size of the testes.

The data obtained are outlined in the tables given below. The age of the bird at the time of the operation, the weight of

the bird at the beginning and end of the experimental period is stated. The weights of the removed, hypertrophied and control glands are expressed in grammes and in per cent. of the body weight of the fowl.

In the group A (Table I.), where unilateral castrations were performed on baby chicks aged one week, six operated birds and

TABLE I.
RESULTS OF UNILATERAL CASTRATION AT ONE WEEK.

No.	Observations at Time of Operation.			Weighings at 32 Weeks.		
	Chick, Weight.	Removed Testis.		Bird.	Surviving Testis.	
		Length.	Width.		Weight.	Per Cent. Weight.
53.....	45.40	R. 3.5	2.0	1,460.80	L. 19.95	1.35
56.....	54.50	L. 4.1	1.2	1,507.50	R. 7.35	0.48
44.....	67.15	R. 4.5	2.0	1,545.85	L. 13.00	0.83
49.....	66.00	L. 5.0	1.3	992.25	R. 0.85	0.095
46.....	57.95	R. 5.5	1.5	1,275.85	L. 18.70	1.46
50.....	50.90	L. 6.0	1.0	1,048.96	R. 5.10	0.49
62c ¹	45.00			1,020.60	R. 0.65	0.063
					L. 0.55	0.053
64c.....	46.50			1,602.55	R. 12.80	0.79
					L. 11.25	0.70
65c.....	47.65			1,219.05	R. 6.95	0.57
					L. 7.50	0.61
63c.....	43.00			1,489.15	R. 8.20	0.55
					L. 7.75	0.52
66c.....	44.00			994.25	R. 6.20	0.61
					L. 5.85	0.58

The measurements given for the testes removed at unilateral castration are in mm. All weights are in grammes. Per cent. weight = percentage of weight of testis to total weight of bird.

¹c = control.

five controls survived for a period of about thirty-one weeks. Unilateral castrations were performed on thirty-five baby chicks and there were a large number of controls; the mortality was due not so much to operative effects but to the very unfavourable weather conditions. Of the six surviving operated birds, three had a left testis while the other three had a right one. Each surviving left gonad is larger than any of the ten control testes; in two of the three cases each surviving left testis is larger than

any of the five control pairs with one slight exception (No.64). These results cannot be due to chance; we are therefore justified in concluding that a surviving left testis, the partner of which is removed at one week after hatching, exhibits a much greater amount of growth than it would have done, approaching in some cases twice the normal growth.

Preliminary histological examinations of sections of hypertrophied testes indicate that all the gonad tissues are equally concerned in this increase in size.

TABLE II.

RESULTS OF UNILATERAL CASTRATION AT 16 WEEKS.

No.	Weighings at Time of Operation.			Later Observations.		
	Bird.	Removed Testis.		Bird.	At 24 Weeks.	
		Weight.	Per Cent. Weight.		Surviving Testis.	
819.....	992.25	R. 0.20	0.020	1,687.60	L. 9.60	0.56
820.....	1,077.35	L. 0.35	0.031	1,630.90	R. 6.35	0.38
826c ¹	1,105.65			1,630.90	R. 8.40	0.50
830c.....	1,162.35			1,602.55	L. 9.80	0.60
					R. 3.00	0.18
					L. 3.10	0.19
At 32 Weeks.						
815.....	1,048.95	R. 0.36	0.033	1,687.60	L. 30.00	1.81
823.....	907.20	L. 0.17	0.018	1,247.40	R. 15.82	1.26
829c.....	1,460.80			2,071.10	R. 14.11	0.66
831c.....	1,048.95			1,857.70	L. 15.78	0.75
					R. 11.82	0.60
					L. 14.11	0.75
At 40 Weeks.						
824.....	1,048.95	L. 0.65	0.061	1,574.20	R. 20.18	1.28
825.....	963.90	L. 0.19	0.19	1,517.50	R. 20.25	1.31
833c.....	992.25			1,574.20	R. 11.61	0.73
					L. 14.30	0.90

All weights are in grammes. Per cent. weight = percentage of weight of testis to total weight of bird.

¹c = control.

The three surviving right testes on the other hand showed no such increase in size, and in fact did not differ significantly from a single control testis.

The second group of young cockerels was castrated at sixteen weeks and the results are tabulated in Table II. The surviving testes were retained eight, sixteen and twenty-four weeks respectively. It was originally planned to observe the degree of compensatory hypertrophy to forty-eight weeks at which time the birds have been fully mature for about sixteen weeks. However a number of birds died owing to one cause and another so that the last data were obtained on cocks aged forty weeks and only on two right testes.

There is no observable compensatory hypertrophy either of the right or the left testis when the glands were retained only for eight weeks after the operation. There was an increase in size during this period but this was identical with the control glands. After a period of sixteen weeks, however, both the left and the right surviving testes show a considerable degree of compensatory hypertrophy, the left testis being heavier than both testes together of each of the two control pairs; the right testis on the other hand, while it exhibited a high percentage weight, was only slightly heavier absolutely than a single control testis. Three birds were available for observation after twenty-four weeks, two of them having a right testis each while the third served as control. Each of the two right testes weighed about 75 per cent. as much as the control pair, the degree of compensatory hypertrophy on a percentage basis being similar to the amount observed after sixteen weeks.

Table III. gives the data for the next group. The cockerels in this group were unilaterally castrated at twenty-four weeks and then observed to forty-eight weeks at intervals of eight, sixteen and twenty-four weeks, respectively. After eight weeks the surviving left testis showed a certain degree of hypertrophy, weighing much more than one of the control pair of gonads and only slightly less than the other. The right testis had not increased at all as compared with the normal. The same is true after sixteen weeks; the left surviving gland is larger than any one testis of the control pairs but not as heavy as one of the

TABLE III.
RESULTS OF UNILATERAL CASTRATION AT 24 WEEKS.

No.	Weighings at Time of Operation.			Later Observations.		
	Bird.	Removed Testis.		Bird.	At 32 Weeks.	
		Weight.	Per Cent. Weight.		Surviving Testis.	Weight.
810....	1,574.20	R. 0.45	0.027	1,801.00	L. 10.90	0.60
801....	1,332.45	L. 0.20	0.016	1,715.95	R. 0.65	0.041
901c ¹				1,574.20	R. 1.50	0.095
902c....				2,241.20	L. 1.31	0.093
					R. 6.65	0.29
					L. 8.95	0.39
					At 40 Weeks.	
813....	1,020.60	R. 0.25	0.024	2,127.80	L. 17.05	0.80
804....	1,545.85	L. 5.0	0.32	2,241.20	R. 8.60	0.37
809c....				2,212.85	R. 12.70	0.57
907c....				2,099.45	L. 14.60	0.65
					R. 7.70	0.36
					L. 8.0	0.38
					At 48 Weeks.	
808 ²	963.90	R. 0.20	0.020	1,162.35	L. 3.18	0.27
812....	1,573.20	L. 2.70	0.17	1,212.85	R. 10.83	0.83
904c....				2,127.80	R. 13.23	0.62
905c....				2,042.75	L. 11.81	0.55
					R. 4.15	0.20
					L. 3.45	0.11

All weights are in grammes. Per cent. weight = percentage of weight of testis to total weight of bird.

¹c = control.

² No. 808 was ill during the first half of the experimental period and lost considerable weight which it had not regained at the time the bird was killed and the surviving testis removed.

pairs, while the right surviving testis is not as heavy as control single testes. After twenty-four weeks there was no hypertrophy at all to be observed in the left surviving testis; as this bird had been in poor condition and lost considerable weight during the experimental period, we do not attach much significance to

this case. The right surviving testis is probably to be regarded as hypertrophied, being heavier than one of the control pairs and its percentage weight greater than any one testis of the other control pair.

In the group where the cocks were mature (Table IV.), there was not so much difference to be observed in the hypertrophy of the surviving testes. The left testis had increased relatively more in size compared with the gland removed at the operation than the right testis. The hypertrophying testes were retained

TABLE IV.

RESULTS OF UNILATERAL CASTRATION AT 32-40 WEEKS.

No.	Weighings at Time of Operation.			Weighings at 40-48 Weeks.		
	Bird.	Removed Testis.		Bird.	Surviving Testis.	
		Weight.	Per Cent. Weight.		Weight.	Per Cent. Weight.
121.....	1,574.20	R. 3.95	0.25	2,042.75	L. 13.87	0.67
114.....	1,517.50	L. 6.55	0.43	1,659.25	R. 14.27	0.86
123c ¹	1,517.50			1,517.50	R. 7.36	0.48
128c.....	1,829.35			1,687.60	L. 7.52	0.49
					R. 5.0	0.39
					L. 4.93	0.39

All weights are in grammes. Per cent. weight = percentage of weight of testis to total weight of bird.

¹c = control.

for only eight weeks, the observation period being planned to extend only to forty-eight weeks of age. The left surviving testis was larger than any single testis of the two control pairs, but not as heavy as either pair together. The right surviving testis was heavier than one control pair and almost equal to the other pair, being 0.86 per cent. of the body weight while the two control testes pairs weighed 0.78 per cent. and 0.97 per cent. respectively. While the cases are few, the evidence seems to indicate compensatory hypertrophy in this group also after a very short period.

IIa. SUMMARY OF RESULTS OF UNILATERAL CASTRATION
AT VARIOUS AGES.

The preceding tables and descriptions demonstrate that removal of one of the testes pair induces an increase in size of the retained partner when the retention period is of sufficient length, with the exception of the right isolated testes of young chicks (Table I.).

When unilateral castration is performed on young cockerels the left surviving gonad hypertrophies to a greater degree than does the right one, the one differing result obtained in No. 808 being due to the bird's general condition.

The increase in weight of the isolated testis becomes manifest only after a certain interval following castration, the interval being apparently somewhat longer for the right hypertrophying testis than for the left one when younger birds are operated upon.

TABLE V.

DATA COMPILED FROM TABLES I.-IV. CONTROL TESTES WEIGHTS
STATED AS AVERAGES.

Age of Bird.		Weight of Isolated Testis.	Average Weight of Controls.	
At Operation.	At Removal.		Testis Pair.	Single Gland.
One week.....	32 weeks	L. 13.00 L. 18.70 L. 19.95 R. 0.85 R. 5.10 R. 7.35	13.54 (5p.)	6.77
16 weeks.....	24 weeks	L. 9.60	12.15 (2p.)	6.07
	32 "	R. 6.35 L. 30.00	27.91 (2p.)	13.98
	40 "	R. 15.82 R. 20.18	25.91 (1p.)	12.85
		R. 20.25		
24 weeks.....	32 weeks	L. 10.90	9.10 (2p.)	4.80
	40 "	R. 0.65 L. 17.05	21.50 (2p.)	10.75
	48 "	R. 8.60 L. 3.18	16.32 (2p.)	8.16
		R. 10.83		
32-40 weeks.....	40-48 weeks	L. 13.87	12.40 (2p.)	6.20
		R. 14.27		

All weights are in grammes.

The latent period is much shorter when adult birds are unilaterally castrated and here the right isolated testis hypertrophies at the same rate and in the single case available even to a slightly greater degree than the left (Table IV.).

Comparison with the averages of the controls rather than with single controls as set forth in the tables increases the probability of these conclusions as examination of Table V. shows. Individual cases emphasize these general conclusions (Table I., Nos. 53, 44, 46. Table II., Nos. 815, 824, 845. Table IV., Nos. 114, 121).

Lipschütz's suggestion for mammals that unilateral castration produces only a more rapid rate of growth rather than a definitive compensatory enlargement of the surviving gonad does not seem a probable interpretation of our results. It would mean that there would be no actual plus in weight of the isolated gonad over one of the control testes pair at the end of the developmental period of the glands. Such a statement requires a definition of the endpoint of growth of the testes, and in view of the normal variation in the weight of the testes as well as the seasonal variation the feasibility of such an absolute determination appears questionable.

The cocks are mature at thirty-two weeks and compensatory hypertrophy as defined is demonstrated not only at this time but as late as forty-eight weeks.

III. NORMAL SIZE RELATIONS OF RIGHT AND LEFT TESTES.

During the course of the experiments we accumulated some data on the size of the right and the left testes of normal cocks. The majority of the observations were made on the gonads of different birds but the records of the control birds in the preceding tables are for pairs. Tables VI. and VII. give the measurements obtained in one week old chicks. The length and width of the right and left testes are given as it was impracticable to secure accurate weights. The weights of the chicks are also stated for comparison. There appears to be a very slight advantage in size on the part of the left testes at this age. In older birds we find such an individual variation occurring in birds of the same age and even of approximately

TABLE VI.

MEASUREMENTS OF LEFT TESTES REMOVED FROM CHICKS AGED ONE WEEK.

No.	Testes.		Weight of Chick.
	Length.	Width.	
51.....	4.4 mm.	1.5 mm.	45.15 gs.
61.....	5.1 "	2.0 "	55.30 "
62.....	4.0 "	1.8 "	45.0 "
63.....	3.5 "	1.1 "	39.30 "
64.....	5.0 "	1.2 "	43.0 "
65.....	4.5 "	2.0 "	46.50 "
66.....	3.0 "	2.0 "	47.65 "
67.....	4.1 "	1.2 "	54.50 "
68.....	4.0 "	1.1 "	40.40 "
69.....	4.0 "	1.5 "	44.0 "
70.....	5.0 "	1.5 "	49.50 "
71.....	5.0 "	1.9 "	44.75 "
72.....	4.8 "	1.9 "	49.90 "
73.....	6.0 "	1.0 "	50.90 "
74.....	5.0 "	1.3 "	66.0 "
75.....	5.3 "	1.3 "	57.85 "
76.....	4.5 "	1.5 "	52.80 "
77.....	5.2 "	1.7 "	57.85 "
78.....	5.0 "	1.3 "	63.80 "

No. of Cases.	Average of Measurements of Testes.	
	Length.	Width.
19.....	4.6 mm.	1.5 mm.

TABLE VII.

MEASUREMENTS OF RIGHT TESTES REMOVED FROM CHICKS AGED ONE WEEK.

No.	Testes.		Weight of Chick.
	Length.	Width.	
52.....	3.5 mm.	1.0 mm.	43.75 gs.
54.....	4.3 "	1.1 "	43.87 "
53.....	3.5 "	2.0 "	45.49 "
55.....	3.0 "	1.5 "	42.60 "
57.....	5.0 "	1.5 "	49.0 "
58.....	4.0 "	1.0 "	43.30 "
59.....	4.0 "	1.3 "	45.60 "
61.....	5.1 "	2.0 "	55.30 "
70.....	4.0 "	1.5 "	39.90 "
46.....	5.5 "	1.5 "	57.95 "
44.....	4.5 "	2.0 "	67.15 "
43.....	6.7 "	1.5 "	56.30 "
42.....	6.0 "	1.5 "	51.10 "
41.....	5.0 "	1.5 "	59.0 "

No. of Cases.	Average of Measurements of Testes.	
	Length.	Width.
15.....	4.5 mm.	1.2 mm.

identical weight, that valid conclusions cannot be drawn from the data obtained in different cocks. The tables compiled are omitted for this reason.

Where testes of one pair were observed as was done for the control cockerels (Tables II.-IV.) the left testes were larger than the right in one case out of two at twenty-four weeks. At thirty-two weeks, the left testes were larger in two out of three pairs, while at forty-eight weeks, the left testis was very slightly heavier (0.01 per cent.) than the right one in one pair; it was smaller than the right testis in two pairs and finally there was one pair in which the gonad weighed exactly the same amount on the left and on the right side.

The tendency of the left testis to be rather larger than the right one in embryonic chicks has been observed by a number of authors. Firket, '14 (4), states that the right testis is noticeably smaller than the left one in the chick at the seventh day of incubation and quotes Semon, '87 (5), as saying that the left testis is much larger at the beginning of its development.

According to Swift, '16 (6), the left embryonic testis is noticeably larger than the right one in the five day chick and the germinal epithelium of the left gonad is also thicker and more extensive. This difference in favor of the left testis is also visible in the six and nine day chick. Riddle, '16 (7), finds no difference between the right and the left testes in common fowl, the age of the birds is not stated.

The greater tendency towards hypertrophy of the left testis discussed under II.*a*, is presumably associated with this embryonic condition, and is of interest in comparison with the very pronounced asymmetry of the female.

IV. DISCUSSION.

From the results described in the preceding pages as well as from the experimental data published by Benoît, the occurrence of compensatory hypertrophy following unilateral castration in young male fowls seems to be well established. We found compensatory hypertrophy of the retained gonad also in adult cocks, differing in this point from Benoît's observations. The period during which the surviving testes were permitted to

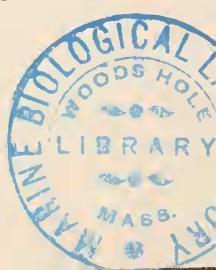
hypertrophy was shorter in our cases than in those reported by Benoît and it is possible that the explanation for the different results obtained may be found in this fact.

The problem of compensatory hypertrophy of the gonad presents its teleological as well as its physiological aspects. The term itself has teleological implications; from this point of view the "purpose" might be either to provide increased reproductive capacity, which appears unnecessary, or to establish a balance of hormones. There again the solution is unsatisfactory for less than one testis is sufficient for maintenance of sex characters as shown by Pézard, '21 (8); '25 (9); Champy, '25 (10).

Physiologically considered it would appear to be obvious that the growth of testis tissue is balanced against something else in the organism.

The general bodily metabolism favors the growth of a definite amount of gonad tissue and no more. The removal of one testis of a pair leaves a balance of conditions favorable to the continued growth beyond its normal size of the surviving member which thereupon responds in proportion to its growth capacity up to the limits of the favorable metabolism. When unilateral castration is performed very early this may result in a single testis greater in weight than a normal pair (Table I., case 16, Table II., case 815), whether there may be a progressive limitation of capacity for compensatory growth with increasing age as maintained by Benoît is still an open question as far as our own results are concerned.

No theory is put forth in explanation of the change in the reaction between gonad and organism which follows removal of one of the gonad pair; it is shown by the facts. But the importance of the principle appears again in the transformations of the female following ovariotomy. The right rudimentary gonad responds with a proliferation of the kind of tissue of which it is composed at the time the demand on it is created, thus producing the various types of right compensatory growth described in completely and incompletely castrated hens by Domm, '24 (11); '27 (12). The principle of compensatory hypertrophy is also illustrated in the growth of grafts.



V. SUMMARY.

1. Unilateral castration in Brown Leghorn cockerels is followed by compensatory hypertrophy of either the right or the left retained testis when the operation is performed on birds aged 16, 24 and 32-40 weeks.
2. The removal of the right or the left testis in chicks aged one week caused a compensatory hypertrophy of the left retained gonads only, after a period of thirty-one weeks, in our experiments (Table I.).
3. There may be a certain period before increase in weight of the retained gonad over the controls becomes manifest; the length of this period is variable.
4. The left retained testis shows a greater tendency towards hypertrophy than does the right.
5. There seems to be a difference in the weight of the left and the right normal testes; this is in favor of the left gonad in very young birds and then gradually seems to become shifted to the right testis as the bird becomes older and reaches maturity.
6. A tentative suggestion is made, that there may be some relation between the greater amount of germinal epithelium in embryonic left testes and the greater tendency towards hypertrophy of the left surviving gonad which is particularly manifest when unilateral castration is performed on young birds.

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NOTE ON THE HEMOLYTIC ACTION OF SEBRIGHT SERUM ON LEGHORN CORPUSCLES.¹

MARY JUHN.

In a series of experiments on grafts of Leghorn testes into Sebright capons and Sebright testes into Leghorn capons carried out by Mr. Roxas in this laboratory (1), the observations were made that the Leghorn testis takes readily in the Sebright but that the inverse is not the case. A much greater mortality was also observed in the Leghorn hosts having Sebright grafts than in the Sebrights having Leghorn grafts. In actual figures:

	Leghorn Testis into Sebrights.	Sebright Testis into Leghorns.
No. of birds.....	38	50
Died from known causes.....	12, 31%	8, 6%
Died, cause unknown.....	8, 21%	24, 48%
Survived to end of exp.....	18, 47%	18, 36%
Birds with succ. grafts (% of surviving birds).....	11, 60%	3, 8%

According to a verbal communication by Mr. Roxas, the Leghorn capons with Sebright grafts that succumbed, showed evidences of pronounced anaemia, this being evident in the lack of color of the headfurnishings which became progressively almost a dead white.

In attempting to determine the causes for the greater mortality among the Leghorn hosts as well as the reason for the much smaller per cent. of successful grafts of Sebright testes into the Leghorns, the following suggestions present themselves. Considering first the conditions in the Sebrights having Leghorn testes grafts, the per cent. of takes is high; the mortality which may be attributed to the graft (cause of death unknown, 21 per cent.) is relatively not so pronounced. We may believe then that the Leghorn tissues are readily incorporated in the

¹ From the Whitman Laboratory of Experimental Zoölogy of the University of Chicago. The expenses of these investigations were supported in part by the Committee for Research in Problems of Sex of the National Research Council; grant administered by F. R. Lillie.

Sebright organism, they soon become vascularized, and being supplied with nutritive substances, persist and even show active growth. There is no extended necrosis in the Leghorn grafts beyond some presumably occurring when it is first implanted and previous to vascularization.

When Sebright testes are implanted into Leghorn tissues however, there is a relatively high percentage of mortality attributable to the graft (cause of death unknown, 48 per cent.) and a very low percentage of takes, 8 per cent. of the surviving birds and only 6 per cent. of all the birds operated upon. There is no evidence of vascularization of the Sebright testis grafts, on the contrary the appearance of the Leghorn capons after Sebright implantations have been made lead one to believe in a greater activity of the lymphocytes and a subsequent elimination of the destroyed graft tissues into the blood stream of the host. Blood counts before and after grafting as well as control of the body temperature would presumably serve to determine the accuracy of these assumptions. The resorption of the graft in a large percentage of cases with subsequent mortality of the host from one cause or another is however apparent.

There is an extensive literature on the subject of the toxicity of organ extracts when injected intraperitoneally, subcutaneously or intravenously. The intravenous injections produce the most rapid lethal effects, but subcutaneous injections of organ paste into guinea pigs, were reported by Brieger and Ulenhuth (2) to kill the animals within 24 hours after the injection. This effect was correlated by Dold and Kodama (3) with the toxic action of tissues in a state of destruction and with the causes of death after burns. Pfeiffer (4) states that in acute cases of death from burns, the cause of the mortality may be traced to toxic poisoning induced by protein fission products. These products appear in excessive quantities owing to the resorptive destruction of the proteins which have been changed and killed through heat.

It appears reasonable in view of the findings in comparable fields reported above, to correlate the percentage of deaths in the Leghorns having Sebright grafts with the non-success of these grafts. The continuous resorption of the graft tissues, may, and probably does, set free into the blood stream of the

host toxic substances resulting from the destruction of the implant, and these toxic substances finally prove fatal to the carrier of the graft.

The point of interest is the reason for the different results obtained in these cross-transplantations. The possibility of some specific differences in the blood of the two breeds of fowl was suggested by Prof. F. R. Lillie; the tests made to establish the presence of such differences are described in the latter part of the paper.

My thanks are due to Professor Lillie both for suggesting the problem reported here and for his continued helpful interest in the work.

Some experiments carried out by Sokoloff (5) serve to illustrate that such conditions can be found in other vertebrates. Sokoloff working on homotransplantations in rabbits found the presence of three types of blood and states that grafts only take when made into animals of identical blood constitution with the donor. When grafts are made into animals where the serum of the host agglutinates the corpuscles of the donor, the graft shows complete necrosis. This destruction of the graft cells leads to the production of specific antibodies in the host which have an unfavorable action on the graft. Any graft will cause the formation of some percentage of antibodies but these are counteracted by the graft as soon as vascularization is established and they then disappear from the blood stream.

Furthermore according to Sokoloff, immunization by intraperitoneal injections of an emulsion of the organ to be grafted causes the appearance of antibodies as well, and the presence of these antibodies inhibits take and growth of the graft.

In studying the conditions in the Leghorns and Sebrights, no tests were made for the determination of circulating antibodies. The sera of Leghorn and Sebright cocks and capons were tested for their agglutinating and hemolytic action on the corpuscles of all four kinds of birds.

The experiments were repeated four times, the preparation of the serum and the corpuscle suspension being identical in every case. For the corpuscles blood was drawn from the ventricle into a syringe moistened with a 1.5 per cent. sodium citrate

solution, 1 cc. of blood was injected into 19 cc. of 1.5 per cent. sodium citrate, the corpuscles then washed four times, centrifuging at low speed to just sediment the corpuscles and the final suspension being brought to 5 per cent. in normal saline. For the serum, blood was run into small test tubes and kept at room temperature over night.

The serum was diluted for all the experiments 1 : 4 with normal saline; the corpuscle suspension being 5 per cent. as stated above. Agglutination tests were made in the hanging drop according to the method outlined by Ascoli (6). No agglutination was observed in any of the serum-corpuscule combinations.

In the tests for the possible hemolytic action of the sera of any one of the four birds, sixteen hemolysis tubes were used in each experiment. Four of the tubes were controls, having the own corpuscles added to the serum, the other twelve were all the possible combinations. One cc. of the 5 per cent. corpuscle suspension was rapidly run into 1.5 cc. of the serum dilution. The tubes were then shaken, placed in the incubator at 38.5° C. for two hours and shaken again every quarter of an hour during this interval. The tubes were then placed in the ice-box at + 9° C. over night and observed the following morning. Furthermore a 5 per cent. corpuscle suspension in saline was always preserved to the end of the period of observation.

The table given demonstrates the results obtained more clearly than any written description. The greater tendency of the Sebright cock and capon serum to hemolyze the Leghorn corpuscles may be noted, but attention must be drawn to the fact, that this is not a constant phenomenon and that in the case of the Sebright capon serum the own corpuscles are hemolyzed to approximately the same degree.

In a single experiment not recorded in the table, the sera of all the birds was diluted 1 : 40 with normal saline. At this degree of dilution distinct hemolysis was observed in the tube having Sebright capon serum and Leghorn capon corpuscles. There was not even a trace of hemolytic action to be noted for any of the other serum-corpuscule combinations.

The different action of the sera tested does not prove an actual difference in the tissues but it is of interest to note the

SERUM.										Leghorn ♂.				Leghorn Capon.			
Sebright ♂.					Sebright Capon.												
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	
Corpusc.	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Sebr. ♂.	—	o	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Sebr. c.	o	++	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Legh. ♂.	++	++	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Legh. c.	++	++	—	—	—	—	—	—	—	—	—	—	—	—	—	—	

1, 2, 3, 4 are the four separate experiments referred to in the text.

o = no tube. — = no hemolysis. +? = very faint traces of hemolysis. + = hemolysis distinct. ++ = hemolysis definite. +++ = entire tube hemolyzed.

parallel between the hemolytic action of some of the Sebright sera on Leghorn corpuscles and the non-success of Sebright grafts in Leghorns except in a very small percentage of cases.

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CONFIGURATIONS OF BIVALENTS OF HYACINTHUS WITH REGARD TO SEGMENTAL INTERCHANGE.

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INTRODUCTION.

In the majority of the flowering plants examined by the writer, and apparently also in most of those investigated in this respect by others, the homologous chromosomes, which form bivalents at the reduction metaphase, are joined only at the extreme ends. As examples, *Canna* and *Datura* may serve, in which this rule holds in the triploids as well as in the diploids. In the largest bivalent of *Uvularia*, however (Belling, 1926), there are additional points of junction (nodes) not at the ends. The short and medium chromosomes of *Uvularia* seem usually to be connected at or near the constriction, and the same is the case with the short and medium chromosomes of *Hyacinthus* (Belling, 1925). These will not be further considered here.

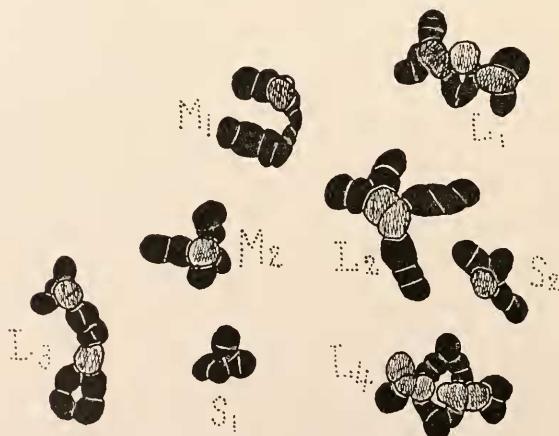


FIG. 1. Camera drawing of the eight bivalents of the diploid hyacinth, squeezed from a pollen-mother-cell. The four large bivalents are alone considered here. They are described in the text.

But the four large bivalents of *Hyacinthus* show, like the large bivalent of *Uvularia*, many connections not at the ends (Fig. 1). It has been pointed out in regard to *Uvularia* (Belling, 1926) that the simplest hypothesis is that these connections (nodes) not at the ends represent places where two of the four chromatids have undergone segmental interchange by fracture and recombination. In *Hyacinthus* it can apparently sometimes be seen with the microscope that two of the four chromatids are bent back at a node, so as to continue along the same sides of the bivalent (Fig. 3). It has also been shown that the homologous chromosomes of the rings and V's formed by the large bivalent of *Uvularia*, acted when separating as if they were not merely twisted across one another, but had undergone a process which produced some interlacing of chromatids at the nodes. This would prevent the simple untwisting of the homologues at the anaphase, and such untwisting has been shown not to occur in *Uvularia* (Belling, 1926). This would also lead (as has been abundantly shown by Janssens, 1924, and others, in animals) to the separation of whole upper and lower halves of vertical rings and V's, and to one chromatid passing up and one down from both sides of horizontal rings or V's, which may get smaller as the process advances, without opening up. This is what takes place in *Uvularia*, and apparently also in *Hyacinthus*.

In *Hyacinthus*, as already stated, the homologues are not always connected at one or both of the extreme ends, but are connected at other places (nodes). This is especially the case with the four long chromosomes. Hence a study of these may show, by the nature of their configurations and their mode of separation at the reduction metaphase, whether the nodes correspond to what would be expected if they were due to segmental interchange between chromosomes (crossing-over of genes).

If the nodes in the long bivalents of the hyacinth are due to segmental interchange, the following phenomena should be observable: (1) the nodes should occur at *different points* in the bivalents in different cases; (2) these nodes should be at equal distances from the ends of both homologues; (3) the nodes should be visible at the late prophase (diakinesis stage or earlier) as well as at the metaphase; (4) the horizontal rings or V's

should split into separate chromatids, while the vertical rings or V's should divide into upper and lower halves, without separating into chromatids; (5) the numbers of bivalents with one or two nodes should permit of a calculation of the numbers of chromatids with segmental interchange at no, one, or two points, which should possibly more or less resemble the occurrence of no, single, or double crossing-over in *Drosophila*. In such a calculation, the numbers of chromatids with no segmental interchange would be equal to twice the cases of single nodes plus the number of cases of double nodes. The total number of chromatids with one point of interchange would be got by adding twice the number of bivalents with single nodes to twice the number with two nodes. While the chromatids with two points of interchange are equal to the number of bivalents with two nodes. It should be possible to test these five points.

(It seems obvious that a junction of homologous chromosomes at the ends has no relation with segmental interchange. It is probably otherwise with junctions at the point of constriction of the chromosome, where segmental interchange may well take place.)

It was for the purpose of testing this hypothesis that the present study was made.

LARGE BIVALENTS OF *Hyacinthus*.

The variety of *Hyacinthus orientalis* investigated was one of those formerly studied (Belling, 1925), and was chosen because it could be readily identified by the flowers, and had marked characters even in the bulbs. This was the diploid clone called "Yellow Hammer." The bulbs were obtained in October, and put into water during that month and the next. Division of the pollen-mother-cells usually accompanied the development of the first roots. The pollen-mother-cells were instantaneously fixed by being squeezed out from the anthers into iron-acetocarmine. The chromosomes were observed with Zeiss' water-immersion objective 70, yellow-green light, and a water-immersion condenser.

At the first metaphase in the pollen-mother-cells (Fig. 1) the four long chromosome pairs commonly assume one of six different

configurations (Diagram I.). Three of these are shown in Fig. 1, where the cross (L_2) is near the center, two single rings with

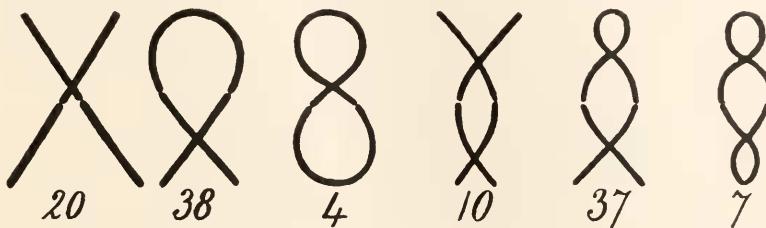


DIAGRAM I. Numbers of different configurations of large bivalents. The cross, single ring and V, and figure of 8, have one node; while the ring and two V's, the double ring and V, and the triple ring have 2 nodes.

double V's are on the right (L_1 and L_4), and a double ring and V on the left (L_3). Four forms in the late prophase are shown in Fig. 2. They are: (a) the ring and V; (b) the double ring

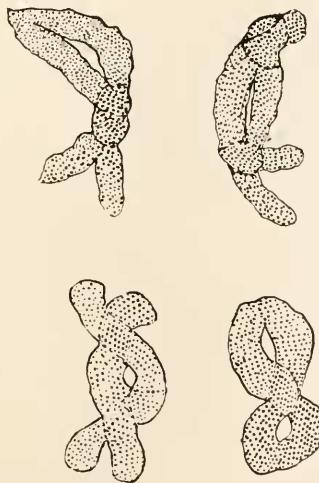


FIG. 2. Four camera drawings of large bivalents at the late prophase. (This stage is difficult to obtain.)

and V; (c) the ring and two V's; and (d) the figure of 8. The cross and the triple ring were not found free, the bivalents being usually clustered at this stage. Several configurations are drawn in Fig. 3. It may be specially noticed that the four examples of the cross shown in Fig. 3 have the junction in different positions with regard to the chromosome ends. The cross in the middle

has arms which taper to the center, the two homologues having apparently joined at the constrictions. The last two drawings in the lowest line of Fig. 3 are the same bivalent at two different focusses, apparently showing one transverse and one reflexed



FIG. 3. Camera drawings of eight bivalents. The four crosses have the nodes at different points, but always at equal distances from the ends of the homologues. The lowest arm of the second cross in the second line is much foreshortened. The last two figures show the effects of change of focus on the node.

chromatid of one homologue, the same thing being often observable at a different focus in the other homologue also. These are pulled out by the spindle fibers which are attached at the apex of the bend at the median constriction.

One hundred and sixteen of the long bivalents were classified in six groups (Diagram 1), mostly after squeezing chromosomes and cytoplasm from the cell. The results were (Diagram 1): 38 cases of the ring and V; 20 X's; and 4 figures of 8 (totalling 62 with one node); 37 cases of the double ring and V; 10 cases of the ring and two V's; and 7 cases of a triple ring; totalling 54 cases with two nodes. No bivalents with triple nodes were certainly demonstrated. If only two chromatids underwent segmental interchange at any one node, as seems to be indicated

by the microscopical phenomena, we have for the resulting pollen grains and any particular long chromosome, on the hypothesis of segmental interchange: no segmental interchange ($124 + 54$), 178; single point of interchange ($124 + 108$), 232; and double interchange, 54; out of a total of 464 chromosomes or pollen grains.

This gives in percentages: chromosomes with no interchange 38 per cent.; chromosomes with single interchange, 50 per cent.; and chromosomes with double interchange, 12 per cent. The ratio of single to double interchange on the hypothesis is thus 4.3 to 1.

This is not far from the numbers of no, single and double points of crossing-over given for the first chromosome of *Drosophila melanogaster* by Morgan (1925). The second and third chromosomes of *Drosophila*, however, seem to differ in this respect.

In Figs. 4 and 5, there are examples of the separation of chromatids and homologues which tend somewhat to prove the hypothesis of segmental interchange. In Fig. 4, L_1 is especially instructive. Here on one side of the constriction (and spindle fiber attachment) there was apparently a horizontal ring, and



FIG. 4. Camera drawing of the bivalents in a cell in which the four large ones had not completely separated, the drawing paper being shifted after each was drawn. Some are foreshortened, especially L_2 and L_4 .

on the other side perhaps a vertical ring and a small V; or only a large vertical V. The horizontal ring evidently split into two ring chromatids, while the vertical ring remains. In L_3 the constriction is in the vertical ring. (L_2 and L_4 show nothing more.) In Fig. 5, L_1 shows apparently the separation of the



FIG. 5. Slightly more advanced stage than Fig. 4.

parts of a cross, the two horizontal members of which can be seen splitting into chromatids, one passing to each pole.

DISCUSSION.

The following points seem most worthy of notice.

(1) At some nodes it appears as if both chromatids of each homologue could be seen, one obliquely transverse, and the other reflexed. The spindle fiber is often attached (at or) near the node also at the median constriction.

(2) Some rather scanty data seem to show in *Hyacinthus*, what is more abundantly demonstrated in *Uvularia*, namely, that single chromatids of each homologue pass to the poles from both sides of horizontal rings and V's, and that the two chromatids of each homologue remain connected in the separate halves of vertical rings and V's. So the horizontal rings and

V's diminish in size as their chromatids are pulled out by the spindle fibers towards the poles.

(3) The numbers of cases of possible segmental interchange in the chromatids of the large bivalents, calculated from the numbers of nodes, agrees roughly with the numbers of cases of crossing-over found in the first chromosome of *Drosophila*, which is especially favorable for this study.

Some process of segmental interchange seems demanded by the genetic evidence in *Drosophila*, *Zea*, *Lathyrus*, and the other plants and animals which have shown cases of crossing-over. Hence it is apparently the natural scientific procedure to accept segmental interchange as a working hypothesis to account for the nodes and internodes of the chromosome pairs in the *Orthoptera* and other animals, and also in *Uvularia* and *Hyacinthus*. This is the more imperative in that there seems no other available working hypothesis.

SUMMARY.

(1) The four large bivalents of *Hyacinthus* show in 62 cases one node, and in 54 cases two nodes where the homologues cross.

(2) At these nodes it can apparently be seen with the microscope that one chromatid of each homologue passes obliquely across, while the other seems bent back along the other homologue.

(3) The hypothesis of previous segmental interchange at such a point is assumed until a better hypothesis is found.

(4) The numbers of chromatids showing such points of segmental interchange, according to the hypothesis, calculated from the 116 bivalents examined, were 38 per cent. with no interchange, 50 per cent. with one point of interchange, and 12 per cent. with two points of interchange.

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